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Planetary Biology and Microbial Ecology: Molecular Ecology and the Global Nitrogen Cycle

Edited by

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Introduction

In the summer of 1991, the NASA Planetary Biology and Molecular Ecology (PBME) program was conducted at the Marine Biological Laboratory (MBL) in Woods Hole, Massachusetts. The program used the facilities of the MBL, including the laboratories, dormitories, cafeteria, and laboratory support system. This was the fifth course in a series of PBME programs, and was only the second to be conducted at a site remote from the NASA Ames Research Center.

The goals of this intensive summer program were twofold: (1) to examine, via lectures and discussions, several aspects of the biogeochemistry of the nitrogen cycle; and (2) to teach the application of modern methods of molecular genetics to field studies. In this sense, this year's program differed from some of the previous PBME courses, which centered more on microbiology. Here we focused on teaching modern approaches to environmental studies of organisms, including not only microbes, but also phylogenetically more advanced forms.

To accomplish the first goal, a distinguished group of scientists was invited to lecture on various aspects of biogeochemistry, specifically the biogeochemistry of nitrogen. The lectures covered many areas, including the biogeochemical cycling of nitrogen in freshwater, estuarine, marine, and terrestrial ecosystems. The lecturers included ecologists, physiologists, biochemists, molecular biologists, and ecosystems scientists. Abstracts of and references for the individual talks are presented in this document.

Of particular note for the goals of the course was the series of lectures given by Dr. David Peterson of the NASA Ames Research Center. These lectures, in combination with those involving stable isotope geochemistry and general biogeochemistry, form a basis for analysis of the nitrogen cycle, and how to relate many of the methods taught here to more broadly based problems.

To accomplish the second goal, both lectures and laboratory exercises were utilized. Three laboratory modules were run consecutively, each an intensive training session of two weeks. These modules were originated and supervised by Dr. Charles Wimpee, Dr. Thomas Chen, and Dr. Dennis Powers, and dealt with molecular biology of bacteria, fish, and mitochondria, respectively. A list of those faculty members and their teaching assistants, directly involved in the laboratory teaching, is included below. Laboratory education consisted of extensive lecture periods discussing both techniques and approaches, coupled with "hands-on" laboratory exercises.

Wimpee Module: Molecular Biology of Bioluminescent Bacteria
Dr. Charles Wimpee, Univ. of Wisconsin-Milwaukee
Assisted by Dr. Daad Saffarini
Assisted by Denise Garvin

Chen Module: cDNA Cloning
Dr. Thomas Chen, Center of Marine Biotechnology, Univ. Maryland
Assisted by Dr. Chun-Mean Lin
Assisted by Michael Shambloott
Assisted by Bih-Ying Yang

Powers Module: Population Polymorphisms and Enzyme Expression
Dr. Douglas Crawford, Univ. of Chicago
Dr. Simona Bartl, Hopkins Marine Station, Stanford Univ.
Assisted by Stephanie Clendennen

Detailed descriptions of the laboratory projects and protocols for each of the three modules are contained in this report. Through this presentation, we have attempted to bring the material to others who might find it valuable.

Previous NASA PBME courses have used somewhat different approaches. They are described in NASA Technical Memorandum #86043 (Carbon Cycling), NASA Technical Memorandum #87570 (Sulfur Cycling), and NASA Contractor Report #4295 (Metal Cycling). Because of the different goals of the laboratory work in this course, the format was quite different than used in previous years. As with the previous courses, however, the experience proved to be exceptionally valuable for the transfer of information and techniques between ecologists, geochemists, and NASA personnel.

MARINE ECOLOGY/PLANETARY BIOLOGY AND MOLECULAR ECOLOGY
Schedule of Lectures and Labs June 16 - July 27, 1991

WEEK I - Module 1

Sunday June 16th	1930	Meet in laboratory Get acquainted session, arrange student talks
Monday June 17th	0830	3 Student Talks
	1000	Dr. Charles Wimpee: Univ. Wisconsin - Milwaukee Nucleic Acid Hybridization
	1300	Dr. Wimpee: Laboratory Technique Lecture Collection and plating of water samples 6 Student Talks
Tuesday June 18th	0830	3 Student Talks
	1000	Dr. Daad Saffarini: Univ. Wisconsin - Milwaukee Laboratory Techniques Lecture
	1300	Isolation and purification of luminous bacteria Colony lifts, lysis, cross-linking Prehybridization of filters Direct PCR of colony lysates, End-polishing Gel purification of PCR products
Wednesday June 19th	0830	3 Student Talks
	1000	Dr. Wimpee: Southern blotting, application of molecular techniques to the study of luminescent organisms
	1300	Inoculate liquid cultures from isolated colonies Make hybridization probes using PCR Hybridize colony lifts
Thursday June 20th	0830	3 Student Talks
	1000	Dr. Saffarini: RNA Isolation and Sequencing
	1300	Restriction digestion, gel electrophoresis Wash primary colony filters and expose Isolate RNA
Friday June 21st	0830	3 Student Talks
	1000	Dr. Kenneth Nealson: Univ. Wisconsin - Milwaukee Biology and Ecology of Luminous Bacteria
	1300	Develop colony films, blot gels, cross-link Prehybridize, hybridize, PCR on genomic DNA End-polishing, gel purification of PCR products Run RNA sequencing gel, dry gels, expose

Saturday	0830	3 Student Talks
June 22nd	1000	Dr. Wimpee: Cloning Strategies and Techniques
	1300	Prepare vector DNA (Phagescript)
		EcoRV digest, phenol extract, EtOH precipitate
		pellet, resuspend
		Spin down, resuspend purified PCR product
		Estimate concentration of vector and PCR product
		Set up ligations, incubate overnight
		Inoculate overnight culture of host cells
Sunday	1300	Free Day (need a few volunteers to
June 23rd		inoculate cells for transformation)

WEEK II

Monday June 24th	1000	Dr. Edward DeLong: Woods Hole Oceanographic Inst. Application of Molecular Genetic Techniques to Microbial Ecology
	1300	Prepare competent cells Plate ligation mixes Inoculate host cells for mini cultures
Tuesday June 25th	0800	Pick plaques, start cultures
	0830	Dr. DeLong: Fluorescent Probe Methodology
	1000	Infect cultures with phage
	1030	Dr. DeLong: Laboratory demonstration and hands on work with fluorescent rRNA probes
	1300	Continue DeLong demonstration, and: Isolate DNA from cultures Run mini lysates on gel; compare size with vector Do c-test to find both strands Prepare sequencing templates
Wednesday June 26th	0830	Dr. Sandra Nierzwicki-Bauer: Rensselaer Polytechnic Inst. Applications of Molecular Techniques to Microbial Ecology, Part 1
	1000	Dr. Nierzwicki-Bauer: Part 2
	1300	Sequence each strand Run sequencing gel Dry, expose overnight
Thursday June 27th	0830	Laboratory All Day
Friday June 28th	0830	Dr. Simon Silver: Univ. Illinois - Chicago Molecular Approaches to Metal Resistance Studies, Pt. 1
	1000	Dr. Silver, Pt. 2
Saturday June 29th	0830	Finish module 1 laboratory work, clean laboratory
Sunday June 30th		Free Day
	1700	Laboratory group meeting and barbecue Meet with Dr. Chen and his group

WEEK III - Module 2

Monday July 1st	0830 Dr. David Peterson: NASA Ames Research Center Remote Sensing of Forest Ecosystems 1000 Isolation of total RNA
Tuesday July 2nd	0830 Dr. Peterson: Remote Sensing and the Nitrogen Cycle 1000 Isolation of poly(A) ⁺ -RNA and agarose gel electrophoresis
Wednesday July 3rd	0830 Dr. Thomas Chen: Center of Marine Biotechnology, University of Maryland I. Structure, Evolution and Regulation of Fish Growth Hormone Genes 1000 II. Transgenic Fish Approaches to Marine Systems 1300 Synthesis of 1st and 2nd strand cDNA, RACE synthesis of cDNA and precipitation of cDNA 2000 Dr. Wimpee: Non-photosynthetic Parasitic Plants
Thursday July 4th	0830 Laboratory Lecture - Dr. Chen 1000 Calculation of cDNA synthesized, methylation, linker I ligation and isolation of genomic DNA from red cells 1800 Volleyball, fireworks at Nobska Point
Friday July 5th	0830 Dr. Thomas Schmidt: Miami University, Ohio I. Molecular Phylogenies and Microbial Evolution 1000 II. Identification Without Cultivation: Using rRNA's to Study Marine Picoplankton 1300 Trimming of excess linkers, precipitation of cDNA Ligation of cDNA to lada Zap vector
Saturday July 6th	0830 <i>In vitro</i> packaging Titer estimation
Sunday July 7th	Free Day (plating out for screening)

WEEK IV

Monday July 8th	0830 Group I. Lifting plaques, labeling probes, prehyb and hybridization Group II. Lifting plaques, blocking, reacting to primary antibody Group III. Digestion of genomic DNA and electrophoresis of DNA
Tuesday July 9th	0830 Dr. Ivan Valiela: Woods Hole Ecosystem Group I. Overview of Nitrogen Cycling in Marine and Aquatic Systems 1000 Dr. Thomas Hollocher: Brandeis Univ. Nitrification and Denitrification 1300 Group I. Wash membranes and expose to X-Ray films Group II. Wash membranes, react to second antibody Group III. Transfer DNA fragments to N/C membranes
Wednesday July 10th	0830 Dr. Joseph Montoya: Harvard University General Overview of Stable Isotopes and Their Use in Biogeochemical Studies, Part 1 1000 Dr. Montoya, Part 2 1300 Group I. Develop films, cork and plate out for second screening Group II. Lift plaques, block and react to primary antibody Group III. Synthesis of radioactive probes, prehyb and hybridize
Thursday July 11th	0830 Millipore Corp. Representative: Sequencing Strategies 1000 Group I. Lift plaques, label probes, prehyb and hybridize Group II. Wash membranes, react to second antibody, develop color Group III. Wash membranes, expose films
Friday July 12th	0830 Dr. Donal Manahan: Univ. Southern California Energy metabolism and animal/chemical interactions 1000 Dr. Manahan, Lecture 2 1300 Group I. Wash membranes, expose to X-ray film Group II. Wash membranes, expose to X-ray film Group III. Develop film
Saturday July 13th	0830 Develop film and discuss results for all groups Clean laboratory area

WEEK V - Module 3

Monday July 15th	0830 Dr. Dennis Powers: Hopkins Marine Stn, Stanford Univ. Overview of Genetic and Biochemical Approaches to the Study of Marine systems, Part 1 1000 Dr. Powers: Part 2 1300 Laboratory Lecture: population markers, PCR primers Get animals, begin genomic DNA prep Discuss designing primers, finish DNA prep
Tuesday July 16th	0830 Determine DNA yields and analyze on gel Go over primer design, examine gels Set up PCR experiments, digest cloning vehicles Prepare next round of PCR samples Start second round of PCRs
Wednesday July 17th	0700 Load gels with PCR samples and digested vectors 0800 Breakfast 0900 Examine gels, clean up PCR products Digest PCR products for cloning Clean up digested vectors, run on low-melt agarose Purify fragments from gel Precipitate PCR products and vector together Set up ligations Transform competent bacteria, plate bacteria
Thursday July 18th	0830 Dr. Daniel Morse: Univ. California - Santa Barbara Ecology and Biology of Larval Settlement 1300 Clean up additional PCR products Digest PCR products for population polymorphism study Start bacterial cultures for ssDNA prep. Gel analyze PCR product polymorphisms Pour sequencing gels for tomorrow
Friday July 19th	0830 Dr. Stephen Macko: Univ. Virginia Use of Bulk Stable Isotopes for Environmental Studies Stable Isotopes at the Molecular Level 1300 ssDNA preps, sequencing reactions, sequencing gels Second loading, soak and dry gel, put down for autorad
Saturday July 20th	0830 Read sequencing gels and analyze data Finish any loose ends, discuss data, clean lab areas
Sunday July 21st	Free Day

WEEK VI

- Monday
July 22nd
- 0830 Dr. Ann Giblin: Woods Hole Ecosystem Center
Nitrogen Cycling in Atlantic Coastal Ecosystems
- 1000 Involvement of Eucaryotic Organisms in the N-Cycle
- 1300 mRNA isolation, prep for centrifugation
Sp6 plasmid digest, pour gel for RNA analysis
Finish RNA prep from centrifuge
Determine yield, gel analyses
- Tuesday
July 23rd
- 0830 Set up Northern, medium, digest p440
Electrophoresis of p440, low melt
Gel purification of p440
Synthesize cRNA from Sp6 vector
Determine cRNA yields
Dot blots
Prehyb. dot blot and northern of LDH probe
Mitochondrial DNA prep
- Wednesday
July 24th
- 0700 Digest mitochondrial DNA
- 0830 Dr. Robert Haselkorn: Univ. Chicago
Regulation of Heterocyst Differentiation in Cyanobacteria
Molecular Biology of Cyanobacteria
- 1300 Gel electrophoresis of mitochondrial DNA
Random prime p440 fragment (2X)
Add label fragment to dot blot, northern
Pressure blot mtc DNA, prehybridize mtc blot
Random prime mtc DNA
Add labeled mtc DNA to Southern
- Thursday
July 25th
- 0830 RNA to cDNA
PCR LDH-B cDNA
Digest with restriction enzymes
Acrylamide gel electrophoresis of cDNA
Electroblot
Prehybridize LDH-B cDNA blot
Wash dot blot and mtc DNA blot
Put filters onto film
Add labeled p440 to cDNA blot

Friday July 26th	0830	Dr. Mitchell Sogin: Marine Biological Labs Recent Developments in the Phylogeny of Eucaryotes
	1300	Cut up dot blot, count activity Wash LDH-B cDNA blot Put on film Develop mtc and cDNA blots Analyze data
Saturday July 27th	0830	Review results Clean up loose ends, clean up lab areas

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Molecular Techniques for the Identification and Study of Luminous Bacteria

**Prepared by: Charles Wimpee, Univ. of Wisconsin-Milwaukee
and Daad Saffarini, Center for Great Lakes Studies, U. WI-Milw.**

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Quick Genomic DNA Isolation from Bacterial Colonies

Ideally, you should start with a plate which has been streaked with a pure culture of bacteria and grown up until the colonies are 1-2 mm.

1. Use an inoculating loop to scrape off a few colonies. Resuspend in 500 μ l of TE buffer, pH 7.5 or 8.
2. Add 20 μ l of lysozyme (10 mg/ml) and 2 μ l of Proteinase K (10 mg/ml).
3. Incubate at 37° for 45 minutes, then add 45 μ l of 20% SDS.
4. Incubate at 60° for 10 minutes.
5. Add one volume of phenol; mix gently. Get rid of protein (layer between aqueous DNA/RNA and phenol).
6. Centrifuge at full speed for 2 minutes in the microfuge. Remove the upper (aqueous) phase to another tube. If aqueous phase is white (dirty), reextract with phenol chloroform (1:1).
7. Extract the aqueous phase with an equal volume of chloroform isoamyl alcohol (24:1). Centrifuge for 1 minute at full speed in the microfuge. Remove the upper phase to another tube.
8. [Optional: add 1/10 volume 3 M KOAc]
Add 2 volumes of cold ethanol. Incubate at -80° for 30 minutes.
9. Centrifuge at 4° for 10 minutes to pellet the nucleic acids.
10. Resuspend the pellet in 200 μ l of TE and add 4 μ l of RNase A (10 mg/ml). Incubate for 15 minutes at 37°.
11. Extract with one volume of phenol/chloroform (1:1). Centrifuge for 1 minute at full speed in the microfuge.
12. Remove the upper phase to another tube, and precipitate the DNA with 2 volumes of cold ethanol.
13. Centrifuge at 4° for 10 minutes to pellet the DNA. Wash the pellet in 70% ethanol, spin again, and dry the pellet briefly.
14. Resuspend the DNA in 40 μ l of TE. Check the yield by running 5 μ l on a minigel to determine how much to use for restriction digests.

Restriction Digest of Bacterial DNA

Generally, a 10X buffer is supplied with restriction enzymes purchased from major manufacturers. A typical reaction mixture consists of:

10X buffer:	2 μ l
DNA + water	17 μ l
enzyme	1 μ l

total volume	20 μ l

Incubate at least 1 hour at the specified temperature. Most restriction enzymes work well at 37°, but consult the manufacturer's instructions (or a good lab manual) for the optimum temperature for a particular enzyme.

At the end of an hour, 0.1 volume of gel loading dye can be added to each sample, and they can be loaded directly on the gel.

Agarose gel electrophoresis

The percentage of agarose used in gel electrophoresis depends on your purposes. In general, the larger the fragments to be separated, the lower the percentage. In our case, we will separate our digested bacterial DNA on 1% gels.

Add the following to a 250 ml Erlenmeyer flask:

- 1 g agarose
- 100 ml TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA)

(TAE buffer is usually made up as a 50X stock from which the 1X working solution can be diluted).

Heat the gel mix to boiling until all of the agarose has melted. (If you use a 125 ml Erlenmeyer for this volume, it is likely to boil over.) This can be done in a microwave oven, in a pan of boiling water, or (if you're a bit more daring) over an open flame. After all the agarose has melted, it is worthwhile to check the volume to make sure you haven't lost a lot during boiling. The graduations on the side of a typical Erlenmeyer flask are accurate enough. It is advisable to cool the agarose to 55-60° C before pouring the gel onto the plate. Many gel plates are made of plastic, and will warp if the agarose is too hot. After cooling the gel, add 10 μ l of a 10 mg/ml solution of ethidium bromide. Swirl to mix, then pour onto the plate. Ethidium bromide is an intercalating dye that fluoresces under UV light, allowing visualization of the DNA. It is a mutagen, so wear gloves while handling it. The same goes for handling the gel later on. Adding ethidium

bromide to the gel before running it is a common practice, but an alternative is to stain the gel after running it by soaking it for 20-30 minutes in a solution of 0.5-1 $\mu\text{g/ml}$ ethidium bromide. Yet another alternative is to add ethidium bromide to the loading dye. Ethidium bromide slightly alters the migration of DNA. In most cases, however, this effect is unimportant. In those cases where it is important, it will be necessary to run the gel without ethidium bromide, and stain it later.

Loading and running the gel

Once the gel has solidified completely, the comb can be carefully pulled out. Pour enough TAE buffer over the gel to fill both reservoirs and submerge the gel under 1-2 mm. It is now ready to load. Suck up the sample (with gel dye added to it) in a micropipettor. Put the pipet tip at the top of a slot and gently push the plunger. The glycerol in the dye will cause the sample to sink to the bottom of the slot. After the gel has been completely loaded, put the top on the gel box, plug in the leads (negative on the end with the slots, positive on the other end). Just remember that DNA is negatively charged, and will migrate toward the positive. If you want to look at the gel the same day, it can be run for a few hours at 100V. If you want to run it overnight, do it at 20V. (These times and voltages apply to a "full size" gel ~15 cm long. "Minigels" run a lot faster.) The gel is usually run until the bromophenol blue is at or near the positive end of the gel. However, if the fragments you are interested in are very small (a few hundred base pairs, for example), you should not run the bromophenol blue all the way to the bottom. After running, the gel is visualized on a UV light box and photographed. Wear goggles.

Southern Blots

The "Southern" blot is named after a guy named Southern. It's a way of transferring the DNA from a gel to a piece of filter membrane, so you can hybridize it with a specific molecular probe. There are lots of variations of the technique, but the one described here is very typical. (By the way, "northerns" and "westerns" are RNA and protein gel blots, respectively.)

Solutions

Depurination solution:
0.25 N HCl

Denaturing solution:
0.5 N NaOH
1.5 M NaCl

Neutralizing solution:
1 M Tris-HCl, pH 7.5
1.5 M NaCl

Procedure:

1. Put the gel in a shallow container (a baking dish or a Rubbermaid container, for example) and pour enough depurination solution in to cover the gel. Soak the gel for 10 minutes, with gentle agitation. (Either put it on a slow shaker or rock it every few minutes). This step is optional, actually. When followed by step 2, it helps break very large DNA fragments into smaller pieces so that they will leave the gel more easily. On small DNA, it isn't necessary, and can actually be detrimental, because the fragments get too small and the bands diffuse.
2. Pour off the depurination solution, and rinse the gel briefly with distilled water. Then pour in enough denaturing solution to cover the gel. Soak for 30 minutes with gentle agitation.
3. Pour off the denaturing solution, rinse briefly, and then pour in enough neutralizing solution to cover the gel. Soak for 15-20 minutes as before, then pour off the solution and add more. Soak another 15-20 minutes. During this time, start setting up the blotting apparatus as described in step 4. That way, when step 3 is finished, you'll be ready to immediately proceed with the blotting.
4. A simple blotting apparatus can be made by putting a sheet of glass or plastic a little bigger than the gel across a shallow container (again, a baking

dish or Rubbermaid container), which acts as a reservoir. Cut a piece of filter paper (e.g., 3MM) as wide as the gel and long enough to reach over the edges of the platform to the bottom of the reservoir. Fill the reservoir with 20X SSC. The filter paper acts as a wick, and the SSC will creep up and across the platform by capillary action. Alternatively, you can wet the filter with SSC using a pipet.

5. Put on a pair of gloves, and cut a piece of filter membrane (such as nitrocellulose, Nytran) the size of the gel. Oil from your fingers will inhibit the wetting of the filter and subsequent binding of the DNA. Wet the filter by laying it in a container of distilled water. Once it is completely wet, pour off the water and pour in enough 20X SSC to cover the filter. The filter is now ready for blotting.

6. When step 3 is finished, the gel can be transferred directly to the blotting platform. Be careful handling the gel. If you break it, simply piece it back together on the platform. A break is unlikely to show on the final autoradiogram if you're careful about fitting the pieces back together. Any areas of the filter paper wick that are not covered by the gel should be covered with plastic wrap or Parafilm to prevent "short circuiting" of the SSC flow.

7. Next, lay the filter membrane over the gel. Look for bubbles under the filter. If you see any, run a gloved finger over the surface to chase the bubbles to the edge.

8. I usually soak a piece of 3MM paper in 20X SSC and lay it over the membrane at this stage, but the step is probably optional. Next, put a stack of paper towels 3-4 inches high on the assemblage.

9. Lastly, put a weight on the stack of paper towels to assure good contact with the membrane. A container with a few hundred mls of water is heavy enough.

10. The blot can be left for several hours to overnight. During this time, the 20X SSC will seep up through the gel and filter and into the stack of paper towels. In the process, the DNA leaves the gel and is bound to the filter, resulting in a replica of the DNA migration pattern.

11. When the blot is finished (after the SSC has soaked 2-3 inches into the stack of paper towels), take off the stack of paper towels (and the extra piece of 3MM, if you used it), but leave the filter membrane on the gel while you mark the positions of the sample wells. You should be able to see the outline of the sample wells through the membrane. They can be marked with a dull pencil. Don't push too hard, or you'll poke holes in the filter.

Marking the wells is essential for measuring the position of the hybridizing bands on the autoradiogram.

12. After the wells are marked, carefully remove the membrane and rinse it briefly by sloshing it in a shallow container of 5X SSC.

13. Allow the filter membrane to air dry, then fix the DNA to it by UV crosslinking (nylon filters) or baking at 80° for 2 hours in a vacuum oven (nitrocellulose). After this step, the filter is ready for prehybridization and hybridization.

Prehybridization of Southern Blots

After the gel has been blotted, the DNA must be fixed to the membrane and the membrane must be prehybridized. With nitrocellulose membranes, fixation is done by baking the filter at 80° for 2 hours in a vacuum oven. Nylon filters (e.g., Nytran) can be fixed the same way, although the vacuum is not required, since nylon doesn't burn up the way nitrocellulose does. Nytran filters can even be fixed on a gel dryer at 80°. The other way of fixing nylon filters is to crosslink the DNA to the membrane using UV light. The latter has the advantage of being very rapid (about 30 seconds instead of 2 hours). In our case, we will UV crosslink.

After crosslinking, the filters are prehybridized. Prehybridization can be thought of as tying up all the non-specific binding sites on the membrane so that our radioactive probe won't stick to the whole filter. The prehybridization solution that we will use is:

- 50% formamide
- 5X SSC
- 10X Denhardt's solution
- 200 µg/ml yeast RNA

The prehybridization solution is made up from stock solutions in the following manner:

For 100 ml:

- 50 ml formamide
- 25 ml 20X SSC
- 20 ml 50X Denhardt's solution
- 2 ml 10 mg/ml yeast RNA
- 3 ml H₂O

Stock Solutions:

50X Denhardt's:

- 1% bovine serum albumin
- 1% Ficoll
- 1% PVP-40 (polyvinylpyrrolidone, avg.mol. wt. 40,000)

20X SSC:

- 3 M sodium chloride
- 0.3 M sodium citrate

There are endless variations of this prehybridization mixture, including mixtures that substitute nonfat dry milk, coffee creamer (e.g. "Cremora"), or even Bailey's Irish Creme for the Denhardt's. There are also some commercial mixes which reputedly produce ultra-rapid results, but I haven't tried them yet. Most people in this field seem to arrive at some mix that produces clean results, and then stick with it.

Prehybridization procedure:

Put the membrane into a sealable plastic bag (i.e., a "seal-a-meal"). At this point, some people pre-wet the filter with water, then pour it off before adding the prehybridization mix. I add the mix directly to the filter without prewetting. It doesn't seem to make any difference. The amount to add is less than you think. A few ml is enough for an average size filter. I usually use 5 to 10 ml for a membrane the size of a full gel (depends on the gel apparatus; mine are about 13 cm x 15 cm). After adding the prehybridization mix, seal the bag and leave it at room temperature for a few hours or overnight. Some people put the bag at the hybridization temperature to prehybridize it, but it is not necessary.

Hybridization of Southern Blots

The hybridization mix is the same as the prehybridization mix, with the addition of 0.2 volumes of 50% dextran sulfate. This is a shortcut which approximates 10% dextran sulfate, and the change in the salt and formamide concentrations does not significantly alter the hybridization conditions. The purpose of the dextran sulfate is to speed up the hybridization, through an excluded volume effect. (A lot of water molecules are tied up with the dextran, so the probe thinks it's in a higher concentration than it really is.)

As often as not, hybridizations are done without the dextran sulfate. With dextran sulfate, I do hybridizations overnight. Without it, I let it go as long as 48 hours, although on occasion I have been in a hurry and have gotten perfectly good signals after 24 hours. If you do not use dextran sulfate, the

probe can simply be denatured and added directly to the prehybridization mix. Contrary to earlier folklore, the prehybridization mix does not have to be replaced. The only reason that I do replace it is that I have gotten into the habit of making a separate mix with dextran sulfate, to which I add the probe. A variation of this is to add both the probe and the appropriate amount of dextran sulfate to the prehybridization mix. All things considered, the latter probably makes the most sense, although I habitually do it the old way.

The probe is denatured by boiling for 3-5 minutes. This is done in an uncapped microfuge tube, or one with a pinhole poked in the top. Actually, the probe will denature above about 94° C, so the water doesn't have to be boiling. You can bring it to a boil, turn off the heat, and put your tube in. In the 3-5 minutes, the temperature is still above the denaturation temperature. An alternative for those who keep a 94° heating block (e.g., for PCR) is to put the probe in the block for 5 minutes.

After denaturation, the probe is pipetted directly into the hybridization mix. You can either (1) add it to a tube, vortex it, and then pipet the tube contents into the hybridization bag, or (2) pipet the probe directly into the bag, seal it, then squish the bag around to mix it.

The hybridization bag is then placed at the hybridization temperature. The temperature you use depends on the homology of the probe and target. For probing the same species, the typical hybridization temperature in 50% formamide and 5X SSC is 42°. This is equivalent to 65° in a completely aqueous buffer. (Formamide lowers the denaturation temperature of nucleic acid duplexes.) For probing a different species, I usually play it safe by hybridizing at room temperature. That will allow a fair amount of mismatch, and non-specific duplexes can be gotten rid of in the washes following hybridization. Contrary to logic, the bag does not need to be agitated.

Washing and Exposing Southern Blots

After hybridization, radioactively labelled probe that is not specifically bound to target DNA must be washed off. The stringency of the wash (i.e., the salt concentration and temperature) is typically higher than the stringency of the hybridization itself. The higher the stringency, the less mismatch can be tolerated. So hybridizations done at relatively low stringency can be washed at high stringency to get clean autoradiograms. On the other hand, if the stringency is too high, you end up washing off the signal from your target sequence. You can make calculations based upon probe length, G+C content, and percent mismatch to determine optimal wash stringencies, but many times (e.g., with cross-species hybridizations) you

don't have all the information (especially about mismatch). For that reason, many investigators determine optimal wash stringencies empirically, using some broad guidelines. In general, an example of a "low" stringency would be 1X SSC, 0.1% SDS at room temperature. A "high" stringency would be 0.1X SSC, 0.1% SDS at 65°. These are the two extremes that I typically use, although it is certainly possible to go lower or higher. Washes done at stringencies between these extremes will show varying levels of signal strength and "noise" level. The washes can be done in the following way:

1. Make up your wash buffer and pour about 100-200 ml into a container such as a glass baking dish or a plastic container like Tupperware or Rubbermaid. An alternative is to do the washes in the same bag or in another bag. A lot of people do this, but I think it's easier to work with an open container instead of cutting open a bag several times. There is an apparatus on the market which uses a reusable bag in which both the hybridization and washes are done. I haven't used it, so I have no opinion about its merits. (I can say that it is expensive, however.)
2. Open the hybridization bag, squeeze out the hybridization mix, and transfer the filter to the dish with the wash buffer. (By the way, you can save the hybridization mix. Contrary to folklore, it can be reused. In this kind of hybridization, very little probe has actually hybridized to your target, and very little has renatured. So you can put it in the freezer and use it on the next filter, as long as you don't wait so long that the radioactivity has dwindled to an unusable level.)
3. Slosh the filter gently in the container for a few seconds to get most of the residual hybridization mix off. Pour off the wash buffer into the liquid waste container, and pour in another 100-200 ml. (You have just gotten rid of most of the unbound counts. Remaining washes will have very few counts/ml, and after the second change, are usually within the legal limit to be put down the drain.)
4. Put the wash container on a slow shaker at the wash temperature. This is usually done in a shaking water bath, because the heat transfer is rapid. If you do it in a dry shaker, it's advisable to preheat the wash buffer to the desired temperature. Of course, if your wash is being done at room temperature, you can just put it on an orbital shaker on the bench top. The shaker should oscillate just fast enough to gently slosh the filter back and forth in the buffer. It's debatable how necessary the agitation is, but it certainly doesn't hurt.
5. Generally two or three wash buffer changes, shaking about 20 minutes for each change, is sufficient. It doesn't seem to hurt anything to overwash the filter. I have frequently left the thing shaking all afternoon.

6. After the final wash, the filter can be taken out, blotted on a paper towel, and sandwiched in Saran Wrap. If you intend to wash the same filter at a higher stringency after the first film exposure, or strip the filter later and hybridize it with some other probe, it is not advisable to let the filter dry out. No filter that I know of can be efficiently rewashed after it is dry, and some types of filters (e.g., Nytran) are very difficult to strip after they are dry. That's why I put it in Saran Wrap immediately. There are a lot of cases where you may want to step up the wash stringency on the same filter, checking the signal at each stringency. So as a general rule, don't let the filter dry.

7. The filter can now be exposed to X-Ray film. I do this by mounting the filter to some sort of backing (a piece of cardboard, an old piece of X-Ray film, etc.) that is the same size as the film, so it won't move around inside the exposure envelope. Usually, you should put some sort of marker on the filter that will expose the film at that spot, allowing you to orient yourself when you look at the developed autoradiogram. Some people use radioactive ink (a little ^{32}P mixed with India ink) or fluorescent ink (which you can buy) and put a little mark at the top of the filter. I use little fluorescent stickers (look in a toy store, or the children's section in a drugstore or card shop. You can also use fluorescent star charts). Expose the sticker to light to charge it up, then stick it to your filter. I use clear tape to do this, so I can reuse the stickers. The fluorescent stickers are much better than radioactive ink, because they give you the same signal, no matter how long or short you do the exposure (the fluorescence dies down after a few minutes). Besides, the less radioactivity you have to keep around the lab, the better. After the filter and its fluorescent marker are ready, put it in an exposure envelope and go into the darkroom. Be careful about the kind of safelight you're using. Be sure it's rated for X-Ray film, or you'll fog your film. If in doubt, do a trial exposure of the safe light on blank film to see if it fogs. Alternatively, it's perfectly possible to do the whole thing in total darkness. Open up the envelope and lay it in front of you. Open the film box, slide out a sheet of film, and put it directly over the filter. Before you do anything else, close the film box. Almost everyone at one time or another forgets to do this before turning the lights on. The result is that the ends of the film get exposed to light, and all of them will lose an inch or two of usable exposure area. (And the film is expensive). Next (if you are using one), put an intensifying screen over the film. (Intensifying screens are optional. They speed up the exposure time.) Close up the envelope and turn on the lights so you can find the doorknob.

8. The exposure can be done at room temperature, but it's faster at -70° , because of reciprocity. In any event, it's usually a good idea to squish the envelope together tightly so the exposure is sharp. If there is any space between the filter and film, the signal comes out fuzzy. This can be done

with a heavy weight like a lead brick or a phone book or some such thing. Alternatively, you can sandwich the envelope between two thick pieces of glass or lucite and clamp them together with large binder clamps (obtainable at a stationery store). A more expensive alternative is to abandon exposure envelopes completely and instead use metal exposure cassettes, which have their own clamps. (Unfortunately, they are currently about \$100 apiece, as opposed to just a few dollars for cardboard exposure envelopes.) The exposure time depends entirely upon how hot the filter is. Some exposures can be done in less than a half hour, while others may go for a week or two. Your best bet if you are unsure is to let it expose overnight. If it's totally overexposed when you develop it the next day, try it again for just a few hours. If you don't see much of a signal, let it go a few days. A weak signal won't be much better unless you substantially increase the exposure time. Doubling the time doesn't necessarily help that much, so I usually at least quadruple the time.

To develop X-Ray Film

- 1) Slide or clip film into hanger (depends on the hanger)
- 2) Submerge in developer for 3 to 4 minutes
- 3) Lift out and drain for 5 to 10 seconds
- 4) Submerge in stop bath for 10 to 20 seconds
- 5) Lift out, drain 5 to 10 seconds
- 6) Submerge in fixer for 3 to 4 minutes
- 7) Lift out, drain 5-10 seconds, run under water in tank for 5-10 minutes
- 8) Hang up to dry

Colony Hybridization

There are several ways of processing membrane filters containing bacterial colonies or phage plaques. The following is probably the most popular. It was developed by Hanahan, and can be found in the Maniatis book.

I find that the procedure of "lifting" the colonies from the plate works better if the plate is cold. 30-60 minutes at 4° is long enough. (If the plate is warm, the colonies have a greater tendency to smear.) Using a gloved hand or a pair of forceps, lay the membrane filter on the plate containing the colonies, making contact with the entire surface and allowing it to wet completely. Nytran wrinkles more easily than nitrocellulose, so try to be careful to either lay down the filter slowly from one edge, or bend it in such a way that the middle of the filter makes contact with the agar first, after which you let the edges go. If the filter wrinkles, don't try to fix it. It isn't a serious problem, and trying to straighten the wrinkle is likely to smear the colonies. However, it's OK to gently run your gloved finger around the surface of the filter to get rid of any bubbles. After it is completely wet (usually 15-30 seconds), lift the filter off of the plate with a pair of forceps. At this stage, you can immediately lyse the colonies and proceed with the prehybridization. Alternatively, you can amplify the colonies by laying the filters, colony side up, on new plates, allowing them to incubate for a few hours. The latter method gives stronger hybridization signals because there is more cell material on the filter after amplifying them.

Colony lysis:

1. Lay out a piece of plastic wrap (e.g., Saran Wrap or Handiwrap).
2. Make a 0.75 ml puddle of 0.5 M NaOH on the plastic wrap, and carefully lay the filter, colony side up, on the puddle. The filter will wet, and the NaOH will lyse the colonies. Leave the filter for 2-3 minutes.
3. Lay the filter on a paper towel to blot it dry (10-20 seconds; it doesn't have to be dry; you just need to get rid of the excess NaOH)). Don't touch the colony side, or you'll smear the lysed colonies.
4. Next, lay the filter on a 0.75 ml puddle of 1 M Tris-HCl (pH 7.4). This neutralizes the filter after the NaOH treatment. Leave the filter for 2-3 minutes.
5. Blot the filter dry as before on a paper towel.
6. Lay the filter on a 0.75 ml puddle of 0.5 M Tris-HCl (pH 7.4), 1.5 M NaCl. Leave for 2-3 minutes.

7. Rinse the filter by immersing it briefly (a few seconds is enough) in a solution of 5X SSC.
8. Blot the filter on a paper towel as before, and then let it air dry completely (30-60 minutes).
9. At this stage, the filter can be processed in exactly the same manner as a gel blot (baking or UV crosslinking, prehybridization, and hybridization).

The Polymerase Chain Reaction (PCR)

This procedure is one of several variations on the basic techniques described by the Perkin-Elmer Cetus Corporation in their kit. There is nothing wrong with buying their kit, but we find that it is cheaper to buy the enzyme and make up our own 10X buffer and dNTP solutions.

This procedure is written with the assumption that the template DNA is present in a concentration of approximately 100 ng/ μ l. A fairly wide range of template concentrations will result in about the same amplification, but this is a case where more is not necessarily better. You can sometimes improve the quality or the yield of a PCR amplification by decreasing the amount of template DNA.

1. Mix together in a 0.5 ml microtube:

H ₂ O	68.5	μ l
10X buffer	10	μ l
primer #1	2	μ l
primer #2	2	μ l
template DNA	1	μ l

2. Mix by flicking the tube with your finger, then spin for a few seconds in the microfuge to bring all the solution to the bottom.

3. Float the tube in a pan of boiling water for 5 minutes.

4. Remove from the boiling water and let cool for 2 minutes at room temperature.

5. While the tube cools, mix together:

16 μ l of dNTP solution
0.5 μ l of Taq Polymerase (2.25 units)

6. Add 16.5 μ l of dNTP/Taq Polymerase to the cooled tube. Flick to mix, and spin briefly in the microfuge to bring the solution to the bottom.

7. Add 3 drops of mineral oil (~100 μ l) with a transfer pipette.

8. Put the tube in the thermal cycler and proceed with the first primer extension (2 minutes at 72°).

9. Continue the cycles as follows:

1.25 minutes at 94°
2 minutes at 37°
2 minutes at 72°

Do this for 28 cycles (for a total of 29 primer extensions). On the 30th primer extension, increase the time to 7 minutes. This is a ridiculously long time, since the polymerase travels at a rate of a least 750 nucleotides per minute. However, it assures that the last primer extension is absolutely complete.

When the 30th cycle is finished, the products can be checked on a minigel. The simplest thing to do at this stage is to put a pipet tip through the oil layer into the aqueous layer, and suck up 5 μ l. Put this drop on a piece of Parafilm, add 1 μ l of gel loading dye, mix by sucking up and down a few times, and load onto a minigel. Alternatively, you can pipet off the oil layer or extract with chloroform to remove the oil. (Remember that chloroform is heavier than water, when you remove the layer.)

Cloning PCR Products into Single-stranded Bacteriophage

There are several ways of getting PCR products into a form that can be sequenced. Direct sequencing is the fastest, and can be done after either symmetrical or asymmetrical amplification. However, although some people have great success with direct sequencing, it can be tricky, and often seems to be template-, primer-, or even strand-specific. We elect to clone the PCR products, which allows us to use universally workable sequencing protocols. The additional time spent cloning (not very long, as you will see) pays off later with the high quality of sequencing reactions that result. In addition, cloning of the PCR products gives you a "hard copy" of the amplified DNA that can be stored and grown up later if you need it. Although it is just as easy to clone into double-stranded vectors, we clone directly into the single-stranded bacteriophage M13 (which can also be isolated in its double-stranded form), because the sequencing reactions we generate are consistently less artifact-ridden. However, it is worthwhile to experience double-stranded sequencing, either of plasmids or PCR products, and to make your own choice, particularly if you foresee doing a lot of sequencing.

When cloning PCR products, the main problem is giving the amplified DNA ligatable ends. This may seem surprising, because we envision the PCR reaction giving nice blunt-ended double-stranded products that, logically, should be easily ligated into a linearized cloning vector. The problem is that apparently only a small percentage of the PCR products in a mixture are truly blunt-ended. There are basically three ways to deal with this:

1. Engineer restriction sites into the primer sequences, so that the PCR products can be digested, generating ends that will ligate to the appropriately digested cloning vector. The trick here is to add some extra

"junk" nucleotides (usually 4 or more) to the 5' ends of the primers. Most restriction enzymes need a little extra DNA outside the actual recognition site to hang onto in order to cut efficiently. This procedure has a lower efficiency than you would logically expect, but we used it for a couple of years with reasonable success, so it's worth considering.

2. The Invitrogen company recently came out with a procedure they call "TA cloning." The ragged ends generated during the PCR reaction are a property of thermostable polymerases, which often add an extra A on the end of the sequence. Invitrogen has taken advantage of this by making a vector with an extra T at the ends, complimentary to the extra A on the amplified DNA. According to their procedure, you simply add your amplified DNA to the already-prepared vector with its T overhang, ligate it, and transform the cells they supply. We tried this out of curiosity and had reasonable success. However, we found that it is almost always necessary to gel-purify the PCR product in order to prevent cloning of shorter and longer "background" products which, although often invisible on a gel, show up with surprising frequency in the resulting clone bank. Another drawback is that the cloning efficiency (by comparison with the procedure we now use) is pretty low. There is a high background of non-recombinant clones, but you can live with the background since the non-recombinants have beta-galactosidase activity which is easily assayed. The kit (which supplies everything you need) is expensive, but it works.

3. "Polish" the ragged ends with a DNA polymerase, which creates good blunt ends that can be easily ligated to a blunt-ended vector. This is the procedure we currently use. The traditional polymerase to use is the Klenow fragment of DNA Polymerase I. However, it has been found (*Biotechniques* 9:710 [1990]) that T4 DNA Polymerase works much better for this procedure. The advantages of the end-polishing procedure are that it is relatively rapid, has (in our hands, at least) much higher cloning efficiency than other techniques we have used, and allows you to clone directly into a vector that produces single-strands if you choose. We use a strain of phage M13 called Phagescript (sold by Stratagene). Although SmaI is a very common enzyme to use for creating blunt ends in the vector, we find that we get higher efficiency if we use the EcoRV site that Phagescript has in its polylinker.

Procedures:

End-polishing and gel purification:

1. After PCR amplification, add 15 units of T4 DNA (about 6 μ l) Polymerase directly to the reaction mix (100 μ l).
2. Incubate 20 minutes at 37°.
3. To gel purify the end-polished PCR product, we use SeaKem agarose (FMC Corporation). The type of agarose you use does make a difference in ligation efficiency. SeaKem is made especially for this purpose. Make a 1% SeaKem agarose gel with 0.5 μ g/ml ethidium bromide. If available, a preparative comb is nice to use. Otherwise, use a standard comb and load several wells.
4. Add 1/10 volume of loading dye (10 μ l) to the PCR mix.
5. Load the whole sample, avoiding the overlaid mineral oil as much as possible.
6. Run the gel at 100V until the PCR product is clearly separated. This will depend on the size of the product and the presence or absence of multiple bands.
7. Cut the band out of the gel with a razor blade, scalpel, plastic gel knife, or even a plastic ruler. Be careful not to cut the gel on a plastic gel plate, if you're using a metal knife. You'll scratch the plate.
8. Rinse previously prepared dialysis tubing with TAE buffer and squeeze out excess.
9. Clamp one end of the tubing (or tie it, which is less convenient) and slide the gel slice into the open end.
10. With a Pasteur pipet or other small pipet, add enough TAE buffer to immerse the slice (usually a few hundred microliters) and squeeze out any bubbles. Clamp the other end of the tubing (or tie it).
11. Put the tubing into the gel apparatus, perpendicular to the current flow (i.e., cross-wise, not length-wise).
12. Turn power supply on at 100V. The elution time depends on the size of the DNA. For small fragments (a few hundred base pairs), 20 minutes is more than enough. Large fragments (kilobases) are slower. Run it for 45

minutes to an hour, and check it on a UV box. Very large fragments don't elute efficiently, so you might lose half of your sample. So try to start with a lot of DNA. Small fragments elute quite efficiently, and we usually don't lose much.

13. At the end of the elution, reverse the current for 45-60 seconds. This pulls the DNA off the wall of the dialysis tubing.

14. Check the elution on a UV light box. If DNA is still in the gel, elute for 10 more minutes on the original direction and check it again.

15. Use a Pasteur pipet to remove the buffer from the tubing and transfer it to a microfuge tube. Rinse the tubing with an extra 100-200 μ l of TAE to remove residual DNA, and transfer it to the same tube.

16. Measure the volume (this can be done easily by weighing the tube and comparing the weight to an empty tube).

17. Extract the eluted DNA with an equal volume of phenol/chloroform. (FMC says that this step is unnecessary with their agarose. They're probably right, but we do it anyway to make sure.)

18. Precipitate the DNA with 1/10 volume of 3 M sodium acetate and 2 volumes of ethanol. We do this at -80° for 15 minutes. This reputedly makes the DNA precipitate more thoroughly.

19. Spin in a cold microfuge for 20 minutes at 10,000 rpm. Wash the pellet with 70% ethanol, then spin in cold microfuge for 5 minutes at 10,000 rpm.

20. Dry the pellet and resuspend in 30 μ l of sterile distilled water.

21. Run 1 μ l of the eluted DNA on a gel along with known amounts of DNA markers in order to determine the concentration of the DNA (4 μ l SDW, 1 μ l XC).

Ligation of polished PCR product to vector:

1. We want a 50:1 molar ratio of insert:vector. The vector (in our case) is M13 Phagescript DNA digested with EcoRV. Cut a known amount of vector DNA with EcoRV, then phenol/chloroform extract, ethanol precipitate, spin, wash pellet, spin, dry, and resuspend. Check the recovery on a gel and adjust the concentration, if necessary. We resuspend the vector at a concentration of 20 ng/ μ l, and the protocol below is written for that concentration. For other concentrations, make appropriate adjustments. By knowing the mass amount and the size of both the vector DNA and the

insert DNA, we can calculate the molar ratio. For example, M13 is around 7.2 kilobases. The luxA fragment we are amplifying is 0.745 kilobases. That's around a ten-fold difference in size (it's safe to approximate these things). To get a 50 fold molar excess of insert (i.e., the amplified luxA fragment), we need a 5 fold mass excess of luxA DNA to M13 DNA.

2. Ligation mixes:

PCR ligation

insert DNA + H ₂ O	10.5	μl	
vector DNA (20 ng/μl)	2.5	μl	[= 50 ng]
5X ligase buffer	4	μl	
10 mM ATP	1	μl	
T4 DNA Ligase	2	μl	

Control ligation

H ₂ O	10.5	μl
vector DNA (20 ng/μl)	2.5	μl
5X ligase buffer	4	μl
10 mM ATP	1	μl
T4 DNA Ligase	2	μl

3. Incubate overnight at 15°.

[stop -- hold at 4°]

4. Run 1 μl of each ligation mix on a minigel to check ligation.

5. Dilute the ligation reactions to 80 μl with water. Reasons: (1) cells begin to saturate with 10-15 ng of DNA, and (2) something in ligation reactions inhibits transformations if not diluted.

Preparation of competent cells:

Have ready:

- YT plates, YT top agar, and YT broth.
 - sterile 50 mM CaCl₂.
 - small sterile culture tubes (e.g., falcon tubes or 100 mm glass culture tubes with caps).
 - 2 heating blocks: one at 42°, one at 50°.
 - the night before, inoculate 1 ml of YT with DH5a cells and shake overnight at 37°.
 - the day they will be used (unless they're warm already), take the YT plates out of the refrigerator and put them in a 37° incubator to warm them up.
- This is optional. We think it helps keep the top agar from gelling before you

get a chance to swirl it around. It's debatable whether warm plates make any difference to the cells themselves.

1. Inoculate 50 ml of YT with 1 ml of DH5a overnight culture. Shake ~2 hours at 37° until $O.D._{600} = 0.3$ to 0.4.
2. While the cells are growing, chill as many 50 ml sterile centrifuge tubes (e.g., "blue caps," "orange caps," or sterile Oakridge tubes with caps) as you need (one per transformation), and 50 ml of sterile 50 mM $CaCl_2$ on ice.
3. Also while cells are growing, melt the top agar and cool it to 50°. We divide top agar into 3 ml aliquots in 100 mm glass culture tubes when we make it up. That way, on the day of the transformation, we melt as many as we need in a beaker of boiling water, then put them in a 50° heating block until they are used. An alternative is to have the top agar in one flask at 50° and pipet it into the transformation mixes (3 ml each) before pouring onto the plates.
4. When the cells are grown up, put them on ice for 20 minutes.
5. Pellet the cells at 5000 x g for 5 minutes in a cold centrifuge (4°).
6. Pour off the supernatant and drain as well as possible, then resuspend in 50 mM $CaCl_2$, 1/2 original volume. Gently swirl to resuspend; the cells are fragile in this solution.
7. Let the resuspended cells sit on ice 20 minutes.
8. Pellet the cells again 5000 x g, 5 minutes. Decant as before.
9. Resuspend the cells in 50 mM $CaCl_2$, 1/20 of original volume, again with gentle swirling. The cells are now competent for transformation.
10. For each transformation, add 300 μ l of competent cells to a chilled sterile culture tube.

Transformation:

Label as many chilled tubes of competent cells as you need. Also, make sure you label all of your plates appropriately, either before or during the transformation, so they will be ready when the transformation is complete.

1. We will transform with 3 different amounts of each ligation mix: 0.1 μ l, 1 μ l, and 10 μ l. We are likely to get the plaque density we want on one of

the lower two dilutions. It's not easy to pipet 0.1 μ l, so take 1 μ l of ligation mix and dilute it (in SDW or TE) to 10 μ l so that you can pipet 1 μ l of it.

2. To chilled tubes containing 300 μ l of competent cells, pipet:

1 μ l of 1/10 diluted ligation mix

1 μ l of ligation mix

10 μ l of ligation mix

Swirl the tubes gently to mix.

3. Return the tubes to ice for 30 minutes.

4. After 30 minutes, heat shock the cells at 42° for 2 minutes. (This is done by moving the tubes to the 42° heating block for 2 minutes.)

5. To each tube, add:

50 μ l 2% X-Gal in dimethylformamide

10 μ l 100 mM IPTG

IPTG is an inducer and X-Gal is an indicator for beta-galactosidase activity. We are cloning into a site that interrupts the beta-galactosidase gene and therefore knocks out its activity. X-Gal turns blue if beta-galactosidase is active; therefore, we are looking for colorless plaques as an indication that our insert DNA is present.

6. Pour 3 ml of 50° top agar into a tube, swirl briefly to mix, then pour onto a YT plate. Gently swirl the plate to spread the top agar. Repeat for all samples.

7. Let the top agar solidify (~10 minutes should be enough), then invert the plates in a 37° incubator and leave them overnight.

8. In anticipation of growing up the clones on the following day, inoculate 1 ml of DH5a cells and shake overnight at 37°.

9. Using sterile Pasteur pipet, pull out isolated plaques (about 10 plaques, 1 per tube). Can store plug in TE at 4°.

10. Blow out into 1.5 ml of DH5a cells in a culture tube. (They were diluted 1/50 from overnight culture.)

11. Shake at 37° for 6 hours.

Preparation of M13 Sequencing Templates

- 1) Use a sterile Pasteur pipet to pull an agar plug containing a single isolated colorless plaque from the plate. Transfer the plug to a 1.5 ml miniculture containing a 1:50 dilution of the overnight culture of host cells (inoculated the night before).
- 2) Shake for 6 hours at 37°.
- 3) Transfer the miniculture to a 1.5 ml microfuge tube and spin out cells for 5 minutes at full speed.
- 4) Carefully (i.e., without stirring up the pellet) transfer 1.2 ml of the supernatant to a fresh tube. Save the pellet and the residual supernatant in the original tube to use as a future inoculum. It can be stored at 4°.
- 5) The supernatant contains the single-stranded M13 phage particles from which you will isolate DNA. Add 300 μ l of 20% PEG (polyethylene glycol)/2.5 M NaCl, vortex briefly, then let sit for 15 minutes at room temperature.
- 6) Spin 15 minutes at full speed in the microfuge. The pellet contains the precipitated phage.
- 7) Carefully pour off the supernatant. The pellet is unstable, and you don't want to lose it. Spin the pellet again briefly and use a pipet to remove any remaining supernatant.
- 8) Resuspend the pellet in 100 μ l of TES by vortexing.
- 9) Extract with 75 μ l phenol/chloroform. Vortex 15-30 seconds, then spin for 2 minutes in the microfuge at full speed. This strips the coat protein from the phage, leaving the single-stranded DNA.
- 10) Pull off the top (aqueous) layer and transfer it to a new tube. Extract it with 75 μ l of chloroform, then spin 2 for minutes at full speed in the microfuge.
- 11) Pull off the supernatant and transfer it to a new tube. Precipitate the DNA with 9 μ l of 3 M sodium acetate plus 200 μ l of ethanol.
- 12) Spin for 20 minutes at 4°.
- 13) Carefully pour off the ethanol supernatant, wash briefly with 300 μ l of 70% ethanol, then spin again for 5 minutes at 4°.

14) Carefully pour off the 70% ethanol and dry the pellet (either air dry or vacuum dry until no visible ethanol is left).

15) Resuspend the pellet in 10 μ l of distilled water. Check the yield by running 1 μ l on a minigel (1 μ l DNA, 4 μ l water, 1 μ l loading dye).

16) The DNA is now ready to sequence.

DNA Sequencing

How to Pour a Sequencing Gel:

This is by no means the only way to pour a sequencing gel. Regardless of which variation you use, however, it is best to pour the gel before you do the sequencing reactions, so that it is ready when your sequencing is done. As you will see, the actual sequencing reactions don't take very long, and you want to make sure you have a good gel ready before you start.

All of the sequencing apparatuses that I know of have a small plate and a large plate. The small plate is a couple of centimeters shorter than the large plate, but the same width. The small plate goes on the inside (i.e., next to the apparatus) when you mount the plate assembly on the gel apparatus. That way, the running buffer from the top reservoir can reach the gel, allowing current to flow. It's obvious when you look at the apparatus.

Before you get involved with assembling the gel plates, it's a good idea to get the gel mix started. It takes a while to go into solution, so it can be stirring while you work on the plates.

Stock solutions:

10X TBE buffer: 0.89 M Tris, pH 8
 0.89 M boric acid
 20 mM EDTA

40% acrylamide/bisacrylamide (29:1)

10% ammonium persulfate (This should be fresh. Some people make it up every day, others every week.)

A standard 8% gel mix:

37.5 g Urea (ultra pure)
7.5 ml 10X TBE
15 ml 40% acrylamide/bisacrylamide
22.5 ml H₂O
Final volume = 75 ml

Stir the gel mix on a magnetic stirring plate. It will go into solution more quickly if you warm it (not hot, just warm).

Now for the gel plates:

1. Good gels start with clean plates. Dirty plates result in bubbles in the gel as the liquid acrylamide is poured between the plates. To clean the plates, use a sponge and some detergent like Alconox or Palmolive. Wash them thoroughly, and rinse excessively to get rid of detergent.

2. Allow the plates to drip dry.

3. At this stage, you need to "waterproof" one of the plates. Specifically, you want to waterproof the inside of the small plate. There are people who will tell you that you don't need to waterproof, but I can guarantee you that you will have fewer gels sticking to plates (disastrous if half the gel sticks to one plate and the other half to the other) if you waterproof. The "old" way to waterproof is to siliconize the plate using silane. It's expensive and you have to use it in the hood. A cheap alternative is to use "Pam" (the stuff you use to keep food from sticking to frying pans). By far the best choice of all is "Rain-X," which you get at an auto supply store for waterproofing your windshield. It's as good as silane, with none of the disadvantages. Procedure: Squirt ethanol on the small plate and wipe it down. Let it dry. Soak a Kimwipe with Rain-X and completely coat one side of the plate (make sure that this becomes the inside of the plate in the finished gel assembly). A hazy film will form, which clears away with longer rubbing.

4. After waterproofing wash both the large and small plates thoroughly with ethanol. Allow the ethanol to evaporate after washing, then repeat about 3 times for each plate (just to be thorough).

5. Wash and ethanol clean the spacers and gel comb.

6. Lay the large plate on a raised surface like a styrofoam box, so you have access to the entire perimeter of it.

7. Place the spacers along the edges and bottom of the large plate. Place the small plate (waterproof side down) on the large plate, align the edges, and adjust the spacer if necessary to make a good seal. Clamp the plates together using binder clips (obtained at an office supply store); 5 small clips along the bottom and 4 large clips along each side works well. Note: An alternative to this is to use only side spacers and to tape the gel plates together using very thin plastic tape. Some people think this is the best technique, but it can be tricky to tape it in such a way that the assembly doesn't leak.

8. When the plate assembly is ready, add 450 μ l of 10% ammonium persulfate to the acrylamide/urea mix.

9. Add 20 μ l of TEMED and immediately pour the gel mix between the plates. The actual pouring can be done using a large syringe, a squirt bottle (e.g., a laboratory wash bottle), or even a small plastic beaker. If you use a beaker, make sure it has a sharp spout on it so that the acrylamide doesn't dribble down the side. Tilt the plate assembly at an angle (~30-45 degrees) and up on one bottom corner, so that the acrylamide flows down to one corner when it is added, and slowly fills up the plate assembly. Pour slowly, watching for bubbles. If a bubble appears, tilt the plate to the other side and pound it out with the side of your hand. If the bubble persists, and it is small, leave it and avoid using that lane when you load the gel. Just circle it with a felt pen on the outside of the plate to remind yourself where it is. If it is disastrously large, try your best to get rid of it. The problem here is that the acrylamide will begin to polymerize in a few minutes, so you don't have a lot of time.

10. Lean the plate back against something so that it is nearly flat, then insert the comb. If you are using a standard comb, push it in until the acrylamide almost reaches the tops of the teeth. If you are using a "shark tooth" comb, insert the comb upside down (i.e., with the teeth up) until the teeth are 1-2 mm above the edge of the larger plate.

11. Clip in 2 places on the top of the gel to press the plates against the comb with no air gaps. (This is a precaution against slightly warped plates, which are not uncommon.)

12. Immediately rinse out the syringe or wash bottle (if you are using one) for future use.

13. When the remaining few milliliters of gel in the bottom of the original container have polymerized (~30 minutes or so), the gel is ready to mount in the apparatus.

14. Gently pull out the bottom spacer (unless you taped the gel plates, in which case there is no bottom spacer). Then pull out the comb. A standard comb can be gently eased out of the gel, after which the wells can be rinsed with 1X TBE buffer and straightened out, if necessary. (Sometimes the wiggly little pieces of polyacrylamide in between the wells get bent out of shape. Be careful straightening them, because they will break.) A shark tooth comb can be gently pulled out and reinserted right side up after the gel is mounted in the apparatus. You might need to clean off bits of dried acrylamide on the outside of the plate, or small bits of polymerized acrylamide from the sample wells.

15. Different apparatuses have different ways of attaching the plate assembly, but most involve some sort of clamping mechanism. After attaching the plate assembly to the apparatus, fill the reservoirs with 1X TBE. After the plate assembly is mounted on the apparatus and the upper and lower reservoirs are filled, you will need to blow out the air space created by the (now removed) bottom spacer. (Again, this is unnecessary if you have used taped plates, because there is no spacer at the bottom.) This procedure can be mildly exasperating, but once you get the knack of it, it is quite easy. It can be done with a Pasteur pipet which has been bent into a U shape using a bunsen burner. Fill the pipet with 1X TBE and position the end so that it is aimed up between the plates on the bottom. Then squirt the 1X TBE out into the space, blowing the bubble toward one side or the other. Keep doing this until you have chased all the air out of the space. The gel is now ready.

Sequencing of Single-Stranded M13 Clones Using "Sequenase" (Modified T7 DNA Polymerase):

Sequencing DNA using the dideoxy chain termination method is conceptually identical to sequencing RNA, although it differs in certain details of the procedure. [NOTE: RNA sequencing is described by Dr. Saffarini in the following section of this publication.] A specific primer is annealed to the template, then the primer is extended with a DNA polymerase, and the growing chains are terminated by addition of dideoxyribonucleotides, resulting in a population of molecules of lengths differing by a single nucleotide. The population of terminated chains can then be separated on a polyacrylamide gel, resulting in a sequencing "ladder" from which the DNA sequence can be read. There are numerous variations on the protocol which follows. This protocol is derived from the one that U.S. Biochemical Corporation provides with its "Sequenase" kit. The procedure involves: (1) primer/template annealing in one tube, (2) a labelling reaction (in the same tube) using only deoxyribonucleotides (including P-labelled dATP) in which primers are allowed to extend for a while before terminating, (3) division of the labelling reaction into 4 separate

tubes (A,C,G,T), (4) addition of the appropriate dideoxynucleotide to each tube to begin terminating chains, and (5) stopping the reactions, followed by denaturation and loading on the gel. The details are as follows:

1. In one micro tube, add:

template DNA (~1 µg) + H ₂ O	7 ml
5X Reaction Buffer	2 ml
0.5 pMole primer (3 ng M13 primer)	1 ml

Mix by gently pipetting up and down a few times.

2. Put tube in 65° heating block for 2 minutes (to denature any interfering secondary structure). To anneal the primer, remove the block itself from the heating element and set it on the bench top to cool to approximately room temperature. (An alternative to this procedure is to put the microtube in a test tube of 65° water, put it in a 65° water bath for 2-3 minutes, then remove the test tube containing the microtube, and let it cool.)

3. While the annealing is taking place:

A. Label 4 micro tubes for the termination reactions. Label one A, another C, another G, another T.

To the A tube, add 2.5 µl ddATP

To the C tube, add 2.5 µl ddCTP

To the G tube, add 2.5 µl ddGTP

To the T tube, add 2.5 µl ddTTP

(These are the tubes that will be used in step 5. Put aside until then.)

B. Make labelling mix dilution in a micro tube. Note: The Sequenase kit comes with a dGTP labelling mix and a dITP labelling mix. The dITP mix is used occasionally to help read through regions of compression (when strings of C's and G's pile up on the sequencing gel). For the usual sequencing reaction, the dGTP labelling mix is used. Both mixes contain dCTP and dTTP. The only difference between the two is that one contains dITP instead of dGTP. The dATP is added as the labelled deoxyribonucleotide.

To read the "normal" range (30-300 nucleotides):

1 µl 5X dGTP mix

4 µl H₂O

To read close to the primer (under ~30 nucleotides):

1 µl 5X dGTP mix

14 µl H₂O

C. Just before you're going to use it in step 4, dilute the enzyme (Sequenase) 1:8 in ice-cold enzyme dilution buffer, and put it on ice. Sequenase will last for about 60 minutes on ice after this dilution is made, so don't do this step until you're ready to set up the labelling reaction.

4. Labelling reaction: Note: this is done in the same tube that the annealing was done in. (i.e., there is only one tube so far). They get divided up into separate A, C, G, and T tubes in the next step.

To the 10 μ l of annealed template/primer, add:

0.01 M DTT (dithiothreitol)	1.0 μ l
labelling mix (diluted in step 3B)	2.0 μ l
32 P-dATP or 35 S-dATP (5 μ Ci)	0.5 μ l
Lastly, add the diluted enzyme:	2.0 μ l

Incubate the labelling mix at 20-25° for 5 minutes. Unless your lab is unusually cold or hot, this is room temperature.

5. Transfer 3.5 μ l of the now-labelled reaction mix to each of the 4 labelled tubes (A, C, G, T) containing the termination mixes (the ddNTP's). Heat 5 minutes at 42°.

6. Stop each reaction with 4 μ l of stop solution. The samples can now be denatured and loaded on the gel.

7. To denature (this separates the labelled ddNTP-terminated strands from the unlabeled template strands): heat the samples to 75-95° for 2 minutes and immediately load on the gel.

8. Loading the gel: you need to blow out the urea which has diffused into the sample wells before loading. Otherwise, the sample will not sink into the well. This is done most easily with a syringe with a small needle, or a Pasteur pipet. Just squirt 1X TBE from the top reservoir into the wells (avoid creating bubbles) to blow out the wells.

9. Run the gel at 2000 volts for the appropriate time depending on your purposes. We routinely run it 2-2.5 hours, turn off the current, load another set of lanes, then run for another 2-2.5 hours before taking the gel off to expose it. That way you get a "short run," allowing you to read near the primer, and a "long run," allowing you to read far into the sequence. If it's a good set of reactions, you can carry this to extremes by running for 8 hours or so. You can get more sequence that way, but not proportionately more sequence, since the migration of the fragments is a logarithmic relationship to fragment length. After a certain length, you can't resolve the fragments no matter how long you run them.

Taking the Gel Off:

This is the most harrowing part of sequencing. You can lose a perfect gel at this point, if you're not careful.

1. Turn off the power and unplug the leads.
2. Unclamp the gel plates from the apparatus and lay the assembly down, small plate up, on the lab bench (preferably on a sheet of benchcoat).
3. Carefully pull out the spacers.
4. Using a thin spatula or other such tool, gently pry the plates apart. If the waterproofing has worked properly, the small plate should come free of the large plate.
5. What you do at this point depends on whether you are exposing the gel wet (an option only if you are using ^{32}P) or dry. The easiest way to deal with a wet gel is to lay a used piece of X-Ray film on top of the gel, make sure it makes good contact with the whole gel surface, and then carefully pick it back up. The gel should come with it. You then lay Saran Wrap over the gel, smooth it out, and take it in the dark room and expose it. If you are going to dry the gel, lay a large piece of filter paper (e.g., 3MM) over the gel and smooth it out. Slowly pick it up from one end, checking underneath to make sure the gel is coming up with it. If it isn't, lay it back down and try again. When you have successfully lifted the gel off the plate, put it on the gel dryer, cover it with Saran Wrap, lay the rubber sheet over it, and turn on the vacuum. It might take both hands (and sometimes more) to hold the rubber sheet down until the vacuum grabs. We use a large piece of plexiglass cut to the size of the gel dryer to hold it down. When the vacuum takes hold, turn the heater to 60-80° and let it dry for a couple of hours. (You can let it go overnight if you don't mind running your vacuum pump that long.) After it's dry, take it in the dark room and expose it. Popular mythology says that you can't expose a ^{35}S gel with Saran Wrap on it. Untrue. Although Saran Wrap decreases the signal from ^{35}S somewhat, it is not a big problem. We use Saran Wrap and easily read them the next morning. The advantage to Saran Wrap is that the film doesn't stick to the gel, which sometimes happens in humid weather. Another popular myth is that you must "fix" gels by soaking them for 10 minutes in 10% methanol, 10% acetic acid before drying them. Here's the real story: Fixing the gel gets rid of the urea. That makes the gel less sticky, so the problem of film sticking in humid weather is lessened. It also makes it easier to get the gel off the plate with 3MM. It is not true that urea kills the ^{35}S signal. Like Saran Wrap, it may lower the signal a bit, but it's not a big problem. We routinely leave the urea in ^{35}S gels. Since we also "break the rules" by leaving the Saran Wrap on, sticking is no problem.

More Comments:

Dideoxy sequencing is generally easier than the alternative (chemical sequencing, also known as "Maxam-Gilbert" sequencing), so it is by far the most popular method. The two techniques have their own set of potential artifacts, so it is hard to say which is "better" overall, although some labs routinely use chemical sequencing for very short fragments that must be read with absolute accuracy. Dideoxy sequencing is best for reading long sequences. Because several primers can be used on the same template (although not at the same time, unless you're using a method that allows you to distinguish between superimposed sequencing ladders), you generally need only one or a few relatively large templates to sequence an entire gene. With the availability of small "consumer-scale" automated synthesizers, the ideal scenario is to sequence a large template until you can't read any farther (about 250-400 nucleotides in an average reaction), then back up about 50 nucleotides and design a new sequencing primer based on the sequence you've just read. The new primer can then be used to read the next 250-400 nucleotides, etc. If you're sequencing cloned templates in any of the commercial plasmid or M13 vectors, the manufacturer can sell you a "universal" sequencing primer which will anneal just outside the polylinker (i.e., the cloning site). Custom internal primers can be made afterward based on the sequence read from the universal primer. If you're sequencing PCR products, you can start with the PCR primers themselves, each in a separate reaction. (Of course, if you're sequencing PCR products which have been cloned into M13, only one PCR primer will work on a given clone, since they're single-stranded.) A cautionary note is that uncloned PCR products are notoriously difficult to sequence and should be meticulously free of the original PCR primers, so that you don't end up priming sequencing reactions in opposite directions at the same time.

Many (but not all) of the artifacts associated with dideoxy sequencing can be attributed to secondary structure in the template or the products. Sequencing artifacts are frequently dealt with by:

1. Using alternative deoxy analogs like dITP or 7-deaza-dGTP, which help prevent compression.
2. Using a different enzyme and/or reaction temperature. Originally, Klenow was the most frequently used enzyme, but Sequenase has gained popularity because it generally reads slightly better. Reverse transcriptase (used at 42-50°) can be used equally well on RNA or DNA templates. It can read through certain regions better partly because of the elevated temperature. More recently, Taq polymerase has been used to help straighten out secondary structure. Because Taq polymerase has an optimum of 72°, most template secondary structure is eliminated. Taq polymerase has also been

used in a "cycling" sequencing reaction which results in linear amplification of the products. The reaction is reminiscent of PCR, but with only one primer.

3. Often, starting with a different primer that still allows you to cover the same region can help read through difficult spots. Better still, reading the other strand (an option available only with DNA) usually takes care of the problem, since opposite strands almost never form the same type of secondary structure. Some journals (for example, *Nucleic Acids Research*) require sequencing of both strands of DNA.

The use of ^{32}P has the advantage of giving you red-hot sequencing ladders that can be exposed and read in a very short time (sometimes 2 hours or less). The gel can be exposed wet, although the bands are correspondingly fuzzier because of the increased distance the beta particle travels before hitting the film. ^{32}P -labelled sequencing products must be loaded on the gel right away (i.e., the same day), because their quality rapidly declines if they are stored. They are noticeably less distinct after as little as one night at -20° . ^{35}S generally gives more readable gels because it yields sharper bands. In addition, the sequencing products can be stored at -20° for days without loss of quality in the bands. This can be a real advantage, since the gels are fragile and occasionally you wreck them in one way or another. When that happens, it's nice to be able to make a new gel the next day and run the samples again. The disadvantage of ^{35}S is that its lower energy requires a dry gel and a longer exposure time than ^{32}P .

In general, the sequencing of single-stranded DNA yields more readable gels than double-stranded DNA. In turn, double-stranded DNA templates generally yield more readable sequences than RNA. PCR products are variable. You may run into people who are able to get gorgeous sequences from double-stranded templates, PCR products, or even RNA, comparable to the quality of single-stranded DNA. But such cases are in the minority, and they are often very sequence-dependent. Some genes just work better than others.

Sequencing technology is advancing at a rapid pace, and I suspect that in 10 years or so, even "small-time" labs will be able to send their templates to "centers" to have them done in automated sequencers. We are almost certainly the only generation who will do this manually. For the present, however, this is what we do. If you foresee doing a lot of sequencing, keep your eyes and ears open for new variations. All of the biotech companies that sell sequencing supplies send out periodical newsletters describing their latest improvements. Some are of dubious worth, but you might run across one that improves the quality or rapidity of your sequencing.

Classification of Bacteria Using 16S rRNA

For the classification of bacteria, sequences of either 5S or 16S rRNA can be used. However, 16S rRNA is longer than the 5S rRNA molecule, and therefore provides more information. Two major methods are currently used for RNA sequences. The first involves the use of 16S PCR products, and the other involves direct sequencing using the reverse transcriptase, which is the protocol that we are going to use.

1- Sequencing RNA using PCR. Two primers are used to generate an almost full length 16S rRNA product. The fragments can then be sequenced either directly or by subcloning into M13. This method is useful when you are trying to identify organisms that cannot be cultured in the lab.

2- Sequencing using reverse transcriptase. Total RNA is extracted and the 16S rRNA is sequenced using primers that are spaced approximately 300 bases apart on the RNA strand. The reverse transcriptase will synthesize a DNA strand that is complementary to the RNA, in a reaction similar to that used in cDNA synthesis. Several primers are used that are approximately 350 bases apart. The sequencing protocol that we are using is the dideoxy sequencing procedure (Sanger and Coulson).

RNA extraction:

- Grow a 50 ml culture overnight.
- Centrifuge the cells at 6000 rpm for 25 minutes. Resuspend the cells in 1 ml of Tris/Mg acetate.
 - 1 mM Tris-HCl
 - 10 mM Mg acetate
 - pH 7.4
- Vortex
- Add an equal volume of 70% phenol (70% phenol, 30% distilled water) to denature the RNase.
- Shake cells vigorously for 10 minutes.
- Centrifuge at 6000 rpm for 10 mins. Aqueous phase (top) contains RNA.
- Transfer top phase to clean tube, and extract once with one volume of TE-saturated phenol/chloroform (1:1). Centrifuge.
- Transfer top phase to a clean tube and extract once with an equal volume of chloroform. Centrifuge.
- To top phase add 1/10 volume 3 M KOAc pH 6.0 and 2 volumes of ethanol. Shake. Keep at -20°C for 1/2 to 1 hour.
- Centrifuge RNA at 8000 rpm for 10 minutes.
- Resuspend in 100 µl DEPC-treated water. Keep at -20°.

Quick Gel:

0.4 g 1% agarose

40 ml TEA buffer

cool to 55°

3 μ l EtBr (Ethidium Bromide)

40 μ l Proteinase K (10 mg/ml)

Use Hind marker; should see three distinct bands 23, 16, and 5.

RNA sequencing:

We are using one primer for sequencing, 1392r. The numbers of the primers correspond to the positions of the primers on the E. coli 16S rRNA.

1. Anneal RNA to primer:

to an RNase free eppendorf tube (0.5 ml) add:

5-7 μ g RNA

1.4 μ l reverse transcriptase hybridization buffer

1 μ l primer (100 ng)

Heat at 85°C for 5 minutes, then transfer immediately to 48°C. Incubate for 45 minutes.

2. Prepare reaction tubes. Label four 0.5 ml eppendorf tubes G, A, T, C. Add 2.5 μ l of the appropriate mix to each tube.

3. To annealing tube add:

3.5 μ l 5X RT buffer

2.5 μ l 32-P-dATP (400 Ci/mMole)

4 μ l RT (diluted 1:5 in RT dilution buffer)

Mix and add 3 μ l to each of the reaction tubes in step 2.

4. Incubate at 50°C for 15 minutes.

5. Add 1 μ l chase mix (2 mM of the 4 deoxys; change ratio of dideoxy:deoxy, thus making it longer) to each tube and incubate further for 15 minutes.

6. Stop reaction by adding 5 μ l of stop buffer. Can hold at -20°. [NOTE: P-32 products degrade after 2 days, S-35 after 1 week]

7. Heat samples at 90°C for 2 minutes to denature, before loading on gel.

Nucleotide Mix:

250 μ M dCTP

250 μ M dGTP

250 μ M dTTP

62 μ M dATP

5X RT buffer to a final concentration of 1X.

Prepare 4 tubes of the above mix and label A, G, T, C.

to A tube add: 50 μ M ddATP

to C tube add: 60 μ M ddCTP

to G tube add: 60 μ M ddGTP

to T tube add: 60 μ M ddTTP

RT hybridization buffer (5X):

0.25M Tris-HCl pH 8.3

0.1 M KCl

RT buffer (5X):

0.25 M Tris-HCl pH 8.5

0.05 M DTT

0.05 M $MgCl_2$

RT dilution buffer:

50 mM Tris-HCl pH 8.3

2 mM DTT

50% glycerol

10X TBE buffer:

1 M Tris-base

1 M boric acid

20 mM EDTA pH 8

Acrylamide/Bisacrylamide:

38 g acrylamide

2 g bis-acrylamide

up to 100 ml water

8% sequencing gel

36 g urea

7.5 ml 10X TBE

15 ml acrylamide

Heat at 50° until melted, then cool at 4° for 15 minutes.

up to 75 ml with distilled water.

450 μ l 10% ammonium persulfate

20 μ l TEMED

[NOTE: on 8% gel: bromphenol blue migrates as if about 20 bases, xylene cyanol ~80 bases. On a 6% gel: migration of bromphenol blue ~40 bases, with xylene cyanol approximately 100 bases.]

Stop Buffer:

45% formamide

20 mM EDTA pH 8.0

0.05% BP Blue

0.05% Xylene cyanol

cDNA Cloning Protocols

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I. EXTRACTION OF TOTAL RNA AND POLY-(A)⁺ -RNA

A. METHOD I: EXTRACTION OF RNA FROM PITUITARIES AND LIVERS OF RAINBOW TROUT BY PHENOL-CHLOROFORM-SDS

1. Mix the following in a RNase-free tube:

1X extraction buffer *	10	ml
10% RNase free SDS	1	ml
Vanadyl Ribonucleosides Complexes	0.5	ml
Polyvinyl Sulfate (25 mg/ml)	20	μ l
Spermine (35 mg/ml)	20	μ l
β -mercaptoethanol (100%)	500	μ l

2. Add 1 gram tissue to the above solution. Homogenize the mixture in an H₂O₂ treated tissumizer.
3. Add 10 ml of phenol saturated with 1X extraction buffer to the tube and shake vigorously for 10 min.
4. Add 10 ml of chloroform and shake for another 10 min at room temperature.
5. Centrifuge at 4000 rpm for 10 min in a table top centrifuge to separate the aqueous and organic phases.
6. Pipet out the organic phase carefully and do not remove the interphase.

_____ aqueous layer (DNA and RNA)

_____ interphase

_____ phenol-chloroform layer

7. Repeat steps 4-6 several times until the interphase becomes minimal (or does not change volume anymore).
8. Add 1/10 vol. of 3 M NaOAc (pH 5.2) and 2.5 vol. of ETOH to the tube. Leave the tube at -20°C for 2 hrs (or overnight).
9. Centrifuge at 10,000 rpm for 10 min at 4°C.
10. Discard supernatant and resuspend the pellet in 10 ml of 3 M NaOAc (pH 5.2). This precipitates the RNA, and the high molecular weight DNA remains in the aqueous phase.

11. Repeat steps 9-10 several times (depending on the amount of glycogen in the sample).
12. After final centrifugation, dissolve the pellet in 5 ml of 20 mM EDTA (pH 8.0) and leave it at room temperature for 10 min.
13. Extract with phenol, then chloroform and precipitate the RNA with ETOH.
14. Rinse with 70% ETOH and dry in the speed vacuum.
15. Resuspend pellet in an appropriate volume of RNase-free H₂O.
16. Make a 1/100 dilution of the RNA solution and determine the concentration in the spectrophotometer. One unit of A₂₆₀ = 40 µg of RNA.
17. Determine the purity of the RNA by running a gel with borate buffer.

NOTE:

*1X RNA extraction buffer:

100 mM NaOAc (pH 5.0)
25 mM NaCl
35 mM MgCl₂
25 mM EGTA

B. METHOD II: GUANIDINIUM THIOCYANATE METHOD FOR RNA EXTRACTION

(a) Reagents:

(1) GT buffer (pH 7.6); the solution must be filtered.

guanidinium thiocyanate	4 M
Tris	20 mM
β -mercaptoethanol	1 M

(NOTE: Add β -mercaptoethanol just before use)

(2) GC buffer (pH 7.0); the solution must be filtered.

Guanidinium hydrochloride	8 M
Sodium acetate	20 mM
Na-EDTA	20 mM

(3) 4 M sodium acetate or potassium acetate (pH 5.2)

(b) RNA extraction:

1. 1 gram of animal tissue in liquid nitrogen powder form is vigorously homogenized in a blender with 10 ml of GT buffer for 2-3 min. The homogenate is centrifuged at 10,000 rpm for 10 min at 4°C. The supernatant is collected in a separate tube.
2. Adjust the supernatant to pH 5.2 with a few drops of concentrated acetic acid. Add 0.5 vol. of 95% ETOH and leave it at -20°C for 30 min.
3. Collect the precipitates by centrifugation at 12,000 rpm for 20 min. Dissolve the pellets in GC buffer (5 ml GC/1 gm fresh tissue).
4. Add 0.5 vol. of ETOH and leave the mixture at -20°C for 30 min.
5. Repeat steps 3 and 4 at least twice.
6. Dissolve the pellets in 20 mM EDTA (2 ml/1 gm tissue) and vortex to resuspend the pellet (you will not get a clear solution at this step). Add 3 vol. of 4 M sodium (or potassium) acetate to the solution (to make the final concentration of the mixture 3 M). The mixture is left at -20°C for 30 min. This step replaces guanidinium salt with sodium or potassium salt in the RNA, and also washes away DNA and tRNA from the RNA sample. Collect the RNA pellet by centrifugation at 12,000 rpm

for 20 min. Repeat this step several times.

7. Dissolve the pellet in RNase-free H₂O. Centrifuge the sample at 8000 rpm for 10 min to remove the undissolved material. The RNA is recovered from the solution by precipitation with 2.5 vol. of cold ETOH and 1/10 vol. of 3 M sodium acetate.
8. Recover the RNA by centrifugation. Wash the pellet in 70% cold ETOH. After drying down the pellet, dissolve the RNA in appropriate volume of RNase-free H₂O. Determine the concentration by spectrophotometer.

C. ISOLATION OF POLY-(A) + RNA BY OLIGO-d(T) COLUMN CHROMATOGRAPHY

(a) Buffers:

(1) 1X binding buffer:

10 mM	HEPES (pH 7.5)
0.5 M	LiCl
1 mM	EDTA
0.1 %	SDS

(2) Elution buffer:

10 mM	HEPES (pH 7.5)
1 mM	EDTA

(b) Procedures:

1. Swell up oligo-d(T) cellulose in 1X binding buffer (1 g/30 ml) at 4°C overnight.
2. Plug the tip of a 1 ml pipette tip with siliconized glass wool, then pipette in about 0.6 ml of oligo-d(T) cellulose.
3. Wash the column with 5 ml of 0.5 N NaOH.
4. Wash the column with 1X binding buffer extensively until the pH of effluent becomes 7.5.
5. Add 1 vol. of 2X binding buffer to the RNA solution, heat at 65°C for 10 min. Allow the RNA solution to cool down.
6. Add the RNA solution onto the column. Collect the effluent. Wash the column with 1 ml of 1X binding buffer. Collect the effluent and combine both effluents together. Heat at 65°C for 10 min and reload onto the column.
7. Collect the effluent and precipitate RNA with ETOH. This fraction would be poly-(A)-RNA.
8. Wash the column with 5 ml of 1X binding buffer and allow the column to drain dry.
9. Elute the column with 2 ml of elution buffer to recover poly-(A) + RNA. Collect 0.5 ml fractions.
10. Add 1/10 vol. of 3 M NaOAc (pH 5.6), 10 µg t-RNA, and 1 ml of ETOH to precipitate the RNA.

11. Wash the RNA pellet with 70% ETOH, dry, and dissolve in 50 μ l of RNase-free H₂O.
12. Run the RNA on 1.2% agarose gel with 5 mM methylmercury hydroxide in the 1X RNA loading buffer*.

(c) Buffers for running RNA gels:

(1) *2X RNA loading buffer:

10 mM	Methylmercury hydroxide (Stock solution is 1M)
2X	Borate buffer
20%	Glycerol
0.03%	Bromophenol blue

(2) 10X Borate buffer (pH 8.2):

Boric Acid	30.9 g/l
Na ₂ -Borate	19.1 g/l
Na ₂ -Sulfate	14.2 g/l

II. CONSTRUCTION OF cDNA LIBRARY

(A) Reagents and Buffers:

- a) 5X Reverse transcriptase buffer (RT buffer):
 - 250 mM Tris-HCl (pH 8.3)
 - 50 mM MgCl₂
- b) 10X dXTP:
 - 12.5 mM dATP
 - 12.5 mM dTTP
 - 12.5 mM dGTP
 - 5 mM dCTP
- c) 5X E coli DNA poll - RNase H buffer:
 - 50 mM HEPES (pH 7.0)
 - 25 mM MgCl₂
 - 500 mM KCl
 - 200 μ M each of dATP, dCTP, dGTP, and dTTP.
- d) 10X Ligation buffer:
 - 500 mM Tris-HCl (pH 7.8)
 - 100 mM MgCl₂
- e) 10X Methylation buffer:
 - 100 mM Tris-HCl (pH 7.5)
 - 2 mM EDTA
- f) 5X T4 polynucleotide kinase buffer:
 - 300 mM Tris-HCl (pH 7.8)
 - 50 mM MgCl₂
 - 60 mM β -mercaptoethanol (β -ME)
- g) 10X Core buffer:
 - 100 mM Tris-HCl (pH 7.5)
 - 100 mM MgCl₂
 - 500 mM KCl
 - 100 μ g/ml nuclease free BSA
- h) Dilution fluid:
 - 10 mM Tris-HCl (pH 7.4)
 - 10 mM MgCl₂
 - 100 mM NaCl

- i) Mg^{2+} - Ca^{2+} solution:
 10 mM $CaCl_2$
 10 mM $MgCl_2$
- j) SAM: Dissolve in 100 mM NaOAc (pH 5.6) to give a solution of 2 mM (i.e. 1 mg/ml). Make a 1/10 dilution from the above solution to give a working solution of 200 μ M.

(B) Synthesis of 1st strand cDNA

- Add 10 μ l poly(A)⁺-RNA and 2 μ g oligo(dT)₁₂₋₁₈
- Incubate at 65°C for 10 min, 37°C for 5 min and on ice for 5 min.
- Add in order:

6 μ l	5X RT buffer
2 μ l	600 mM β -ME
3 μ l	10X dXTP
2 μ l	α - P^{32} -dCTP
30 U	RNasin
50 U	AMV Reverse transcriptase (25 U/ μ l)
- Add H₂O to a final volume of 30 μ l
- Incubate at 42°C for 30 min, then on ice for 5 min

(C) Synthesis of 2nd strand cDNA

To the tube from (B), add:

20 μ l	5X <u>E. coli</u> DNA pol I - RNase H buffer
25 U	<u>E. coli</u> DNA polymerase I
1 U	RNase H

- Add H₂O to a final volume of 100 μ l
- incubate the reaction mixture at 15°C for 2 hrs
- add 10 μ l of 0.1 M EDTA and 40 μ l H₂O
- phenol extraction once (with equal volume of phenol)
- Chloroform-Isoamyl-Alcohol (CIA) (24:1) extraction once
- spin dialysis through a Sephacryl-300 column
- add 10 μ g tRNA, 1/10 vol. of 3 M NaOAc (pH 5.6)
- add 2 volume ETOH
- keep at -20°C overnight
- centrifuge in microfuge for 15 min
- wash the pellet once with 70% ETOH
- dry, dissolve the pellet in 40 μ l H₂O

- take 1 μ l for radioactivity counting
- calculate the amount of cDNA synthesized
- aliquot 4 μ l to be run on 1.2% agarose gel to analyze second strand synthesis quality

(D) Blunting the cDNA termini

To the remaining 35 μ l, add:

- 5 μ l 100 mM Tris-HCl (pH 7.5)
- 5 μ l 250 μ M dATP, dGTP, dCTP, dTTP each
- 5 U Klenow enzyme (1U/ μ l)

- incubate at 37°C for 30 min
- add 50 μ l of H₂O to the tube
- extract the sample with an equal volume of phenol:chloroform
- precipitate the cDNA with 0.3 M NaOAc and ETOH at -80°C for at least 30 min
- spin the sample at 4°C for 30-60 min
- decant the supernatant very careful to another clean tube
- use Geiger counter to monitor the radioactivity both in the supernatant and the pellet. You should have majority of radioactivity in the pellet.
- dry the sample
- dissolve the pellet in 20 μ l of H₂O

(E) Methylation of cDNA

- To the double stranded cDNA from (D) add:

- 15 μ l 10X methylation buffer
- 15 μ l 200 μ M SAM (to give a final concentration of 20 μ M)
- 300 U EcoRI methylase

- add H₂O to a final volume of 150 μ l
- incubate at 37°C for 30 min
- add 12 μ l of 200 μ M SAM and 120 U EcoRI methylase
- incubate at 37°C for 30 min
- add 50 μ l TE buffer (10 mM Tris-HCl pH 7.5, 1 mM EDTA)
- phenol extraction once
- CIA extraction once
- adjust to 0.3 M NaOAc
- add 2 volume of ETOH
- keep at -20°C overnight or dry ice for 30 min.

- wash the pellet with 70% ETOH
- dry the pellets and dissolve it in 20 μ l H₂O

(F) Ligation of linkers

- Add:
 - 20 μ l double stranded cDNA
 - 5 μ l 10X ligation buffer
 - 4 μ l EcoRI linkers (phosphorylated)
(*100X excess of linkers)
 - 5 μ l β -ME (600 mM)
 - 5 U T4 ligase
 - 5 μ l 10 mM ATP (pH 7.0)
- add H₂O to a final volume of 50 μ l
- incubate at 15°C overnight

(G) Trimming of excess linkers

- Add:
 - 50 μ l (from ligation reaction in F)
 - 6 μ l 10X core buffer
 - μ l EcoRI (50 - 100 U)
- add H₂O to a final volume of 60 μ l
- incubate at 37°C for 2 hr
- heat at 65°C for 10 min
- add 40 μ l TE buffer
- phenol extraction once
- spin dialysis through a Sephacryl-300 column
- adjusted to 0.3 M NaOAc, precipitate with ETOH (2 volume)
- on dry ice for 30 min
- centrifuge in microfuge for 15 min
- wash pellet with 70% ETOH, dry
- dissolve cDNA in 5 μ l H₂O
- aliquot 1 μ l for radioactivity counting to reestimate the quantity of ds-cDNA

(H) Ligation of cDNA to lambda vector's arms

- set up 2X ligation mix:

2 μ l 10 mM ATP
2 μ l 10X ligation buffer
2 μ l 600 mM β -ME
10 U T4 ligase
- μ l H₂O to a final volume of 20 μ l

- set up ligation reaction:

1.5 μ l cDNA (to give an equal molar ratio with
vector arms)
1.0 μ l vector arms (1 μ g/ μ l)
2.5 μ l 2X ligation mix

- add H₂O to a final volume of 5 μ l
- incubate at 15°C overnight

(I) In vitro packaging

- thaw out the Freeze/Thaw extract (red tube) between two fingers, return the extract to ice
- thaw out the Sonic extract (yellow tube) between two fingers and return to ice
- add the Freeze/Thaw extract to the ligated DNA solution, and keep the tube on ice
- transfer 15 μ l of Sonic extract to DNA-Freeze/Thaw mixture
- mix the contents well, incubate at 22°C for 2 hours (Do Not Vortex)
- add 0.5 ml of lambda phage dilution fluid and 20 μ l chloroform

(J) Titration

To estimate the titer of the in vitro packaged recombinant phage, E. coli C600 hfl⁻ is used as indicator cells. For amplification of the library, the same host cells are used as well.

- grow E. coli C600 hfl⁻ and C600 in L-broth containing 0.2% maltose overnight
- collect the cells by centrifugation
- resuspend the cells in 1/10 of original volume of 10 mM MgSO₄ solution (this is called plating cells)
- dilute the phage with lambda dilution fluid to 1/100
- set up the following tubes:

- a) 0.1 ml plating cells (C-600) + 0.1 ml diluted phage
- b) 0.1 ml plating cells (C-600) + 0.1 ml diluted phage
- c) 0.1 ml plating cells (C-600) + 0.01 ml diluted phage
- d) 0.1 ml plating cells (C-600) + 0.01 ml diluted phage
- e) 0.1 ml plating cells (C-600 hfl⁻) + 0.1 ml diluted phage
- f) 0.1 ml plating cells (C-600 hfl⁻) + 0.1 ml diluted phage
- g) 0.1 ml plating cells (C-600 hfl⁻) + 0.01 ml diluted phage
- h) 0.1 ml plating cells (C-600 hfl⁻) + 0.01 ml diluted phage

- incubate at 37°C for 15 min
- add 3 ml of 0.7% soft agar to each tube, overlay the mixture on LB-plate
- incubate at 37°C overnight

(K) Amplification of cDNA Library

- to be discussed in class

(L) - A brief description of plaque hybridization by blotting

Preparation of plating cells:

- incubate overnight at 37°C, 1 loopful of E. coli C600 hfl⁻ culture to 200 ml L-broth containing 0.2% maltose
- centrifuge down the cells at 6000 rpm for 10 min
- resuspend the cells in 20 ml of 10 mM MgSO₄. The plating cells are good for up to 3 weeks at 4°C.

Plating out the Library:

- LB-plates should be very dry so that the soft agar (0.7% agarose in L-broth) adheres well to the top of the plates.

L-broth:

Bactotrypton	10g	
Yeast extract	10g	add H ₂ O to 1000 ml
NaCl	10g	

- you need to plate out 30 large plates (150 mm). Each plate contains about 3×10^4 PFU (total 1×10^6 PFU)
- to each tube, add:
 - 0.2 ml plating cells
 - appropriate volume of diluted phage
- incubate at 37°C for 15 min
- add 10 ml soft agarose (0.7% in L-broth), overlay on plate
- incubate the plate at 37°C overnight

Blotting:

- keep the plates at 4°C for at least one hour
- lay down a 137 mm nylon disc membrane on the plate for 30 seconds to 1 min. At this time, use a syringe needle to punch three holes through the membrane in order to align the orientation.
- each phage containing N/C filter is then treated as follows:
 - lay down membranes with phage side facing up on a stack of 3MM papers saturated with 0.5 N NaOH - 1.5 M solution for 1 min. Repeat once.
 - neutralize in 0.5 M Tris-HCl - 1.5 M NaCl (pH 7.5) for 5 min (make sure filter is well neutralized)
 - soak in 6X SSC for 5 min
 - blot dry, UV cross-linked
 - hybridization: under standard conditions

CALCULATION OF AMOUNT OF ds-cDNA SYNTHESIZED

- 1) Amount of cold dCTP used per reaction:

$$5 \text{ mM} \times 3 \mu\text{l} + 0.2 \text{ mM} \times 20 \mu\text{l} = 19 \text{ nmole}$$

- 2) Amount of α -P³²-dCTP used per reaction:

$$0.0033 \mu\text{mole/ml} \times 2 \mu\text{l} = 6.6 \text{ (pmole)}$$

- 3) Dilution factor = $\frac{19,000 + 6.6}{6.6} = 2879$

- 4) Specific activity of α -P³²-dCTP (undiluted)

$$3000 \text{ Ci/mmole} = 3 \mu\text{Ci/pmole}$$

$$1 \mu\text{Ci} = 2.2 \times 10^6 \text{ CPM}$$

$$2.2 \times 10^6 \text{ CPM} \times 3 \mu\text{Ci/pmole} = 6.6 \times 10^6 \text{ CPM/pmole}$$

After dilution:

$$\frac{6.6 \times 10^6 \text{ CPM}}{2879} = 2292 \text{ cpm/pmole}$$

- 5) Amount of ds cDNA synthesized

$$\frac{\text{Total CPM} \times 4 \times 330}{2292} = \text{pg}$$

III. PREPARING HIGH SPECIFIC ACTIVITY SINGLE-STRANDED cDNA PROBES

A. Stock solutions: (all solutions must be RNase-free)

1.	dATP, dGTP, dTTP	4 mM each
2.	Tris-HCl (pH 8.3)	1 M
3.	DTT	0.2 M
4.	KCl	1 M
5.	oligo-d(T)	1 mg/ml
6.	Actinomycin D	1 mg/ml (in ETOH)
7.	MgCl ₂	160 mM
8.	NaOH	1 M
9.	HCl	1 M
10.	EDTA (pH 8.0)	100 mM

B. Preparation of 2X reaction mixture:

	<u>Volume</u>	<u>Final concentration</u>
Actinomycin D	36 μ l	dry in speed vac.
dATP	50 μ l	200 μ M
dGTP	50 μ l	200 μ M
dTTP	50 μ l	200 μ M
Tris-HCl	50 μ l	50 mM
DTT	100 μ l	20 mM
KCl	100 μ l	96 mM
MgCl ₂	38 μ l	6 mM
oligo-d(T)	4 μ l	5 μ g
H ₂ O	58 μ l	
Total	500 μ l	

C. Setting up reactions:

1. To each tube, add:

- 50 μ l 2X reaction mixture
 - 10 μ l poly-(A) + RNA (one pass through oligo-d(T) column)
 - 10 μ l α -³²P dCTP (specific activity 3000 Ci/mmmole)
 - 27 μ l RNase-free H₂O
 - 1 μ l RNasin (30 units/ μ l)
 - 2 μ l Reverse transcriptase (25 units/ μ l)
- add H₂O to a final volume of 100 μ l

2. Incubate at 42°C for 1 hour.
3. After incubation, add 10 μ l of 100 mM EDTA to stop the reaction.
4. Add 10 μ l of 1 N NaOH to the tube and incubate at 65°C for another 20 min.
5. Add 10 μ l of HCl to the reaction tube.
6. Pass the reaction mix through a G-50 spin column. Aliquot 1 μ l for scintillation counting to determine the specific activity of the probe.
7. Boil the probe for 2 min immediately before adding to the hybridization solution.

NOTE: If lower than 30% of the total radioactivity added to the reaction is incorporated into cDNA, the resulting cDNA will not be a good probe.

IV. TRANSFER OF NUCLEIC ACIDS TO NYLON MEMBRANE AND
CONDITIONS FOR HYBRIDIZATION

A. TRANSFER OF DNA OR RNA TO NYLON MEMBRANE

1. DNA fragments are fractionated on 1-3% agarose gel, and stained with EtBr.
2. Gel is treated with 250 ml of 0.25 M HCl for 15 min with gentle agitation. Repeat once. Then rinse with H₂O.
3. Denaturation:
Gel is soaked in 250 ml of 0.5 N NaOH, 1.5 M NaCl solution for 15 min. Repeat once.
4. Neutralization:
After denaturation, gel is soaked in 250 ml of 1 M NH₄OAc, 0.02 N NaOH solution for 30 min, repeat once.
5. Blotting (layers top to bottom):

_____	_____ Paper towel (3 inches)
_____	_____ 3MM Paper (3 sheets)
_____	_____ Nylon membrane
_____	_____ Gel
_____	_____ Nylon membrane
_____	_____ 3MM Paper (3 sheets)
_____	_____ Paper towel (3 inches)

6. Let transfer go for 1-2 hrs.
7. After transfer, nylon membrane is washed with 2X SSC solution for 10 min.

8. Place the membrane into the Stratalinker UV crosslinker while the membrane is still damp, but not dripping wet.
9. Use auto crosslink mode to UV cross-link nucleic acids onto the membrane.
10. Wrap with aluminum foil and store in dry place.

Reference:

G.E. Smith, and M.D. Summers (1980). The bidirectional transfer of DNA and RNA to nitrocellulose or diazobenzylloxymethyl paper. *Analytical Biochemistry* **109**:123-129.

B. HYBRIDIZATION CONDITIONS

(A) Conditions of nucleic acid hybridization depend on the type of probes being used:

1. DNA fragment probes prepared from nick-translation or random priming method:

6X SSC
50% Formamide
5X Denhardt's
0.1% SDS
100 μ g/ml denatured calf thymus DNA
 32 P-labeled DNA (at least 2×10^6 cpm/ml)

Incubate at 42°C overnight

2. Probes prepared from end-labeled oligomers:

6X SSC
5X Denhardt's
0.1% SDS
0.05% Na-pyrophosphate
End-labeled oligonucleotide

Hybridization temperature is 5-7°C below T_d (dissociation temperature), which is estimated by the following equation:

$$T_d = 4 \times (G + C) + 2 \times (A + T)$$

(B) Washing Conditions:

1. Wash filters at room temperature with 4 changes of solution of 2X SSC and 0.1% SDS, 5 min each.
2. Wash filters at 42°C with 2 changes of 1X SSC and 0.1% SDS, 30 min each.
3. Blot dry and expose to X-ray film.

V. DNA SEQUENCING WITH T7 DNA POLYMERASE (SEQUENASE)

1. Annealing mixture:

ssDNA(0.05 μ g/ μ l)	5 μ l
H ₂ O	3 μ l
10X buffer	1 μ l
primer(0.5 pmole/ μ l)	1 μ l
total	10 μ l

Anneal by heating at 80°C for 2 min and cool slowly at 37°C for 30 min.

2. While cooling, label four tubes as G, A, T, and C and fill each tube with 2.5 μ l of each termination mixture ddG, ddA, ddT, and ddC, respectively.
3. Dilute enough T7 DNA polymerase (Sequenase) (1:8) for all templates in icy cold TE buffer.
4. Labeling reaction: To annealed DNA mixture (step 1), add the following:

0.1 M DTT	1 μ l
1X labeling mix	2 μ l
S ³⁵ or P ³² dATP	1 μ l
diluted Sequenase	2 μ l

Mix and incubate at room temperature for 5-10 min.

5. Transfer 3.5 μ l of labeling reaction mix (step 4) into each termination tube (step 2), mix and incubate at 37°C for 5 min.
6. Stop the reaction by adding 5 μ l of stop solution (0.03% bromophenol blue; 0.03% xylene cyanol FF in 100% formamide).
7. Heat samples to 80°C for 2 min and immediately load into the 6% sequencing gel.

NOTE:

(A) For sequencing double-stranded plasmid DNA, please follow this protocol

1. Denaturation:

DNA	5 μ l (1-2 μ g)
2 N NaOH + 2mM EDTA	2 μ l
H ₂ O	15 μ l
Total	22 μ l

Mix and incubate at room temperature for 5 min.

2. Neutralization:

Add 8 μ l 1 M Tris-HCl (pH 4.5)
3 μ l 3 M NaOAc (pH 5.6)
75 μ l ETOH

Leave it at -80°C for 10 min, and spin down DNA.

3. Dry down DNA sample and resuspend it in 7 μ l of water. Add 1 μ l of primer and 2 μ l of 5X buffer, and incubate at 37°C for 15 min.

4. Then, continue from step 2 of previous section.

(B) Sequencing of single stranded DNA templates prepared by Asymmetric PCR amplification

1. Design amplification primers at least 200 base pairs 5' to the site of sequencing primer.
2. Carry out PCR amplification using 1:50 ratio of amplification primers.
3. After PCR amplification, add 10 μ l of 3 M NaOAc (pH 5.2) and 200 μ l isopropanol to each sample. The samples are mixed and allowed to sit for 30 min at room temperature, then pelleted by centrifuge for 30 min at room temperature, washed once with 0.5 ml 70% ETOH and dried.
THIS IS CRITICAL IN ORDER TO REMOVE EXCESS AMOUNTS OF AMPLIFICATION PRIMERS.
4. Resuspend the DNA in 40 μ l of TE buffer and be ready for sequencing.

(C) Sequencing of double stranded DNA templates prepared by Symmetric PCR amplification

1. After PCR amplification, clean up products by GENE CLEAN kit if you have a single DNA band on the gel. Otherwise, run an agarose gel and slice out the band you are interested in and purify it through GENE CLEAN kit.
2. Boil the DNA sample with primer in the 1X sequencing buffer for 5 min, then leave the tube in either liquid nitrogen or ETOH-dry ice bath for 3 min.
3. Keep the frozen tube in the -20°C heat block insert and leave it there until the insert returns to the room temperature.
4. The tube is now ready for sequencing reaction using the Sequenase kit.

(D) Reagents of Sequenase kit:

1. 5X Sequenase buffer:

200 mM Tris-HCl (pH 7.5)
100 mM MgCl₂
250 mM NaCl

2. 5X Labeling Mix:

7.5 μ M dGTP
7.5 μ M dCTP
7.5 μ M dTTP

3. Termination Mix:

	ddG	ddA	ddT	ddC
dGTP	80 μ M	80 μ M	80 μ M	80 μ M
dATP	80 μ M	80 μ M	80 μ M	80 μ M
dTTP	80 μ M	80 μ M	80 μ M	80 μ M
dCTP	80 μ M	80 μ M	80 μ M	80 μ M
ddGTP	8 μ M			
ddATP		8 μ M		
ddTTP			8 μ M	
ddCTP				8 μ M
NaCl	50 mM	50 mM	50 mM	50 mM

4. Stop Solution:

95% Formamide
20 mM EDTA
0.05% Bromphenol Blue
0.05% Xylene Cyanol FF

5. 6% Sequencing Gel:

20 ml 30% Acrylamide Solution
(29:1 Acrylamide:Bis-Acrylamide)
42 g Urea (final concentration is 7 M)
10 ml 10X TBE buffer (0.89 M Tris-HCl,
0.89 M Boric Acid, 2 mM EDTA,
pH 8.3)
0.3 ml 10% Ammonium Persulfate
25 μ l TEMED
-- ml H₂O
100 ml final volume

(E) Conditions of running Gel (used with 0.4 mm wedge spacers):

1. Pre-run sequencing gel at 1200 volts (constant voltage) for 1/2 to 1 hour.
2. Run samples at 1450 volts for 2 hours and 45 min for the first loading. The xylene cyano FF dye should be 27 cm down from the wells.
3. Run the second loading for another 1 hour and 45 min and stop the gel. You should be able to read the very first base of the sequence.
4. In order to resolve 600 to 800 bases from the sequencing reactions using M13 single stranded DNA as templates, please use the following modifications to obtain the best results:
 - (a) Run a 6% sequencing gel at 1200 volts (constant voltage) for 16 to 18 hours. **Be sure gel is not leaking!**
 - (b) Run an 8% sequencing gel at 1200 volts for 8 hours, 4 hours per loading.

VI. POLYMERASE CHAIN REACTION ASSAY CONDITIONS

1. Recommended working dilutions:

Note: Use sterile, siliconized microfuge tubes.

- A. dNTPs Mix: Prepare a mix of dNTPs, 1.25 mM (in each dNTP) final concentration.

Example:

125 μ l	dATP 10 mM
125 μ l	dCTP 10 mM
125 μ l	dGTP 10 mM
125 μ l	dTTP 10 mM
500 μ l	Double-distilled, sterile water
1000 μ l	dNTPs mix, 1.25 mM (in each dNTP) final concentration

- B. Control template: Prepare 10-fold serial dilutions of the control template in 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0, 10 mM NaCl. (Do at least a 10^{-1} dilution).
- C. Depending on the sample DNA preparation procedure, the suggested concentration of Mg^{++} may not be optimal for an efficient amplification. It may be advisable to initially conduct a Mg^{++} titration experiment in the presence of an excess amount of Taq Polymerase (i.e., 4 units of enzyme/sample). Once the optimum Mg^{++} concentration for a given DNA sample is determined, it is recommended to carry out a Taq Polymerase titration in order to optimize the amount of enzyme required for a maximum amplification efficiency.

Note: Briefly (about 2 seconds) vortex, then spin down (quick spin with the tabletop microcentrifuge) the enzyme before pipetting. Use extreme caution and proceed slowly with the pipetting. The enzyme storage buffer contains 50% glycerol and errors can be easily introduced by hasty pipetting. If possible, use a positive displacement pipetting device.

2. Reaction Mix (per assay):

Note: Use sterile, siliconized microfuge tubes. This is the standard protocol for the control template. Overlay the samples (100 μ l in 0.5 capped polypropylene tubes) with 100 μ l of mineral oil (such as Sigma Cat. No. 400-5 or M-3516) to prevent evaporation. The oil layer will not interfere when withdrawing aliquots from the sample for analysis. If the whole sample (100 μ l) needs to be recovered, extract the sample with 100 μ l of chloroform (high purity grade). The aqueous phase, containing the sample, will form a micelle near the meniscus. It will then be easier to collect the sample by withdrawing it with an Eppendorf pipette. Larger master mixes can be prepared and aliquoted before adding the template and the Taq Polymerase. If you suspect that your template is contaminated with proteases (as may be the case with genomic DNA), add the Taq Polymerase after the initial denaturation step. The high temperature incubation should inactivate proteases and prevent degradation of the Taq Polymerase.

3. Experimental Protocol:

Note: Performance of the PCR technique with the GeneAmp™ DNA Amplification Reagent Kit, including Taq Polymerase, can be automated with the **PERKIN ELMER CETUS** DNA Thermal Cycler. The instrument enhances the user's ability to control and optimize the PCR technique to achieve maximum amplification efficiency. Operating instructions and protocol recommendations for use with the DNA Thermal Cycler are contained in the Operator Manual. The following manual procedure is recommended for those using heat blocks or water baths. Use heat blocks or water baths (one each) adjusted to the following temperatures: 37°C, 72°C, and 94°C. Initial template denaturation step: 1 min. 30 sec. at 94°C. Afterwards, a typical cycle profile will be:

- 2 minutes at 37°C (annealing)
- 3 minutes at 72°C (extension)
- 1 minute at 94°C (denaturation)

Repeat for a total of 24 additional cycles. At the end of the 25th cycle, **OMIT** the heat denaturation step and extend the extension step by an additional 7 minutes.

Following the termination of the assay, let the samples come back down to room temperature. The samples can be kept at +4°C overnight, pending further analysis.

- Note:
- A. The size of the target sequence in your sample DNA will directly impact the minimum time required for proper extension (72°C incubation step). An optimization of the temperature cycling profile should be performed for each template, in order to obtain maximum amplification efficiency.
 - B. Be aware that there is a significant lag time (30 seconds to 1 minute) between the time tubes are transferred from one temperature to another and the time the samples actually reach the new set temperature. The lag time will be a function of the type of heat source and tubes used, the sample volume and the temperature differential between incubation steps. Adjust the length of the incubation steps to compensate for this lag time. (Microfuge tubes from Robbins Scientific of Mountain View, CA, Cat. No. 1048, appear to provide the most satisfactory performance.)

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1. Saiki, R.K., S. Scharf, F. Faloona, K.B. Mullis, G.T. Horn, H.A. Erlich, and N. Arnheim (1985) Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* **230**:1250-1354.
2. Scharf, S.J., G.T. Horn, and H.A. Erlich (1986) Direct cloning and sequence analysis of enzymatically amplified genomic sequences. *Science* **233**:1076-1078.
3. Chien, A., D.B. Edgar, and J.M. Trela (1976) Deoxyribonucleic Acid Polymerase from the extreme thermophile Thermus aquaticus. *J. Bacteriol.* **127**(3):1550-1557.

VII. PREPARATION OF SINGLE-STRANDED cDNA SYNTHESIS FOR PCR

Synthesis of 1st strand cDNA:

- Add 10 μ l poly(A)⁺ - RNA and 2 μ g oligo(dT)₁₂₋₁₈
- Incubate at 65°C for 10 min, 37°C for 5 min and on ice for 5 min.
- Add in order:

6 μ l	5X RT buffer
2 μ l	600 mM β -ME
3 μ l	10X dXTP
30 U	RNasin
20 U	AMV Reverse transcriptase
- Add H₂O to a final volume of 30 μ l
- Incubate at 37°C for 1 hour, then on ice for 5 min.
- Do ETOH precipitation as usual, ie, add 1/10 vol. of 3 M NaOAc (pH 5.6) and 2.5 vol. of ETOH to the tube and keep it in -80°C for 15 min.
- Spin in microfuge for 15 min, dry the pellet and resuspend it in 100 μ l sterile H₂O
- Aliquot 1 μ l for PCR amplifications

VIII. CLONING OF DNA FROM PCR AMPLIFICATION

1. After PCR amplifications, run a 1.2% agarose gel to examine the products. If there is only one band with the expected size DNA, then transfer whole reaction mixture to a clean 1.5 ml eppendorf tube and proceed to step 2. If there are multiple bands on the gel, then run a preparative gel, slice out the fragment you are interested in, and continue with the following:
2. Add 3 vol. of NaI solution (from GENECLAN kit) and 30 μ l of glassmilk (from GENECLAN kit) to the tube, mix well and keep it on ice for 10 min.
3. Spin in microfuge for 30 seconds. Discard the supernatant.
4. Add 400 μ l of NEWWASH solution to the tube and resuspend it well by vortexing.
5. Quick spin in the microfuge and decant the supernatant.
6. Repeat steps 4 and 5 twice.
7. Add 50 μ l of H₂O to the tube and incubate at 50°C for 5 min.
8. Spin in the microfuge for 1 min and save the supernatant.
9. Aliquot 10 μ l of DNA from step 8, and add 13 μ l H₂O, 3 μ l of 10X Kinase buffer, 3 μ l of 10 mM ATP (pH=7.0), and 1 μ l of T₄ kinase (10 units/ μ l) to a final volume of 30 μ l. Incubate at 37°C for 1 hour.

10. Add 3 μ l of 3 M NaOAc (pH = 5.6) and 75 μ l of ETOH to precipitate DNA.
11. Resuspend DNA in 11 μ l of H₂O, and set up the following for filling in and ligations:

DNA (in H ₂ O)	11 μ l
10X ligase buffer	2 μ l
Klenow (2 units/ μ l)	1 μ l
dNTPs (from PCR)	2 μ l

Incubate at 15°C for 1 hour. Then, add 1 μ l of blunt-end vector DNA (0.1 μ g), 2 μ l of 10 mM ATP (pH = 7.0) and 1 μ l of T₄ ligase (1 unit/ μ l) to the same tube and continue incubation overnight.

12. Carry out transformation as usual.

STRATEGIES FOR CLONING AND CHARACTERIZING cDNA

I. Introduction

(i). Differences between cDNA and genomic genes

Complementary DNA (cDNA) is synthesized *in vitro* from mature mRNA by reverse transcription. Hence, the sequence of a cDNA is complementary to that of its corresponding mRNA. In other words, the cDNA sequence of an mRNA contains only sequence of the 5' untranslated region, the translated region, and the 3' untranslated regions of the mRNA molecule.

A genomic gene contains the 5' control region, the transcribed region, and 3' flanking regions of a gene. In the transcribed region there are exons and introns. Therefore, the main difference between a cDNA sequence and its corresponding genomic gene is that the cDNA sequence contains neither introns nor the 5' and 3' flanking regions of the gene.

(ii). Purposes of cloning cDNA and genomic genes

(a). A cDNA can be used for the following studies:

- serving as a probe for the isolation of a genomic gene
- serving as a probe for studying the expression of a gene
- studying the primary sequence of a protein
- for large scale production of biosynthetic proteins in microorganisms

(b). A genomic gene is routinely used in the following studies:

- studying the structure and evolution of a gene
- elucidating the regulation of expression of a gene at the molecular level
- for gene transfer studies

II. Messenger RNA Population in Eukaryotic Cells

(i). There are numerous species of mRNA molecules present in any eukaryotic cell. Some of these mRNA species are present in high abundance, while others are present in very low frequency. For instance:

	mRNA species	molecules/cell
(a). mouse liver	9	12,000
	700	300
	11,500	15 (35%)
(b). chick oviduct	1	100,000
	7	4,000
	12,500	5 (32%)

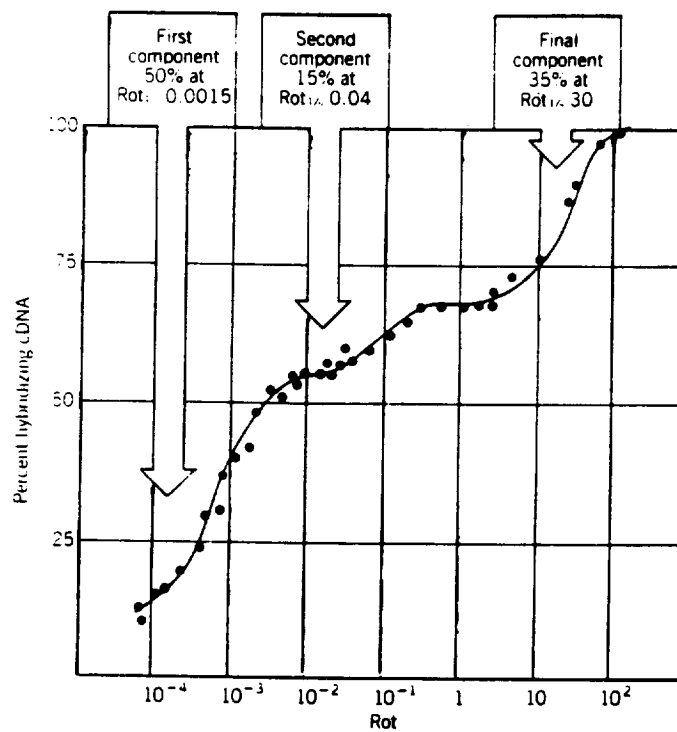
The number of mRNA species present in any eukaryotic cell and their relative frequencies can be estimated via analysis of the hybridization kinetics between mRNAs and their corresponding cDNAs. This analysis is called "Rot Analysis."

The following example is the Rot analysis of the mRNA population in estrogen-treated chick oviduct. In this analysis, total mRNA is isolated from estrogen-treated chick oviduct, reverse transcribed into cDNA and hybridized back to its mRNA (Fig.1). The kinetics of hybridization between cDNA and mRNA is measured and the results plotted as % of hybridization verses initial concentration of mRNA times time (Rot). From the analysis of the hybridization kinetics it is clear that there are at least three different mRNA populations with different frequencies present in the estrogen-treated chick oviduct. The complexity of each mRNA population can be estimated by comparing the Rot_{1/2} (the Rot at which 50% of the mRNA has hybridized to its cDNA) of each unknown mRNA population with that of a known mRNA under the identical hybridization conditions. The complexity of an unknown mRNA can be determined by the following relationship.

$$\frac{\text{Rot}_{1/2} \text{ of the unknown mRNA}}{\text{Rot}_{1/2} \text{ of the known mRNA}} = \frac{\text{Complexity of the unknown mRNA}}{\text{Complexity of the known mRNA}}$$

If the Rot_{1/2} and the complexity of ovalbumin under the same hybridization conditions are determined to be 0.0008 and 1.9×10^3 , respectively, then:

$$\text{Complexity of [A] population} = \frac{0.0015 \times 0.5 \times (1.9 \times 10^3)}{0.0008} = 1.7 \text{ Kb}$$



Hybridization between excess mRNA and cDNA identifies several components in oviduct cells, each characterized by the Rot_{1/2} of reaction.

Fig.1

$$\text{Complexity of [B] population} = \frac{0.04 \times 0.15 \times (1.9 \times 10^3)}{0.0008} = 14 \text{ Kb}$$

$$\text{Complexity of [C] population} = \frac{30 \times 0.35 \times (1.9 \times 10^3)}{0.0008} = 25000 \text{ Kb}$$

If the average size of the mRNA in any population is about 2 Kb, then there are 7 (14 Kb/2 Kb) different mRNA species in class [B] population, and 12500 (25000 Kb/2 Kb) different mRNA species in class [C] population. The average number of molecules of each mRNA per cell is called abundance or representation. It can be calculated quite simply if the total mass of RNA in a cell is known, since for each component, total mass = abundance x complexity, so that:

$$\text{Abundance} = \frac{\text{gm of mRNA in cell} \times \text{fraction in component} \times (6 \times 10^{23})}{\text{Complexity of component in daltons}}$$

In the example above, there are 0.275 pg mRNA per cell. This corresponds to 100,000 copies of the first component mRNA, 4,000 copies of each of 7-8 mRNAs in the second component, and about 5 copies of the 12,500 mRNAs in the third component.

(ii). cDNA library: a collection of cDNA clones that gives a fair representation of all mRNA sequences present in a cell. In constructing a cDNA library, effort is made to prepare sufficient amount of cDNA molecules to will give a fair representation of all the mRNA species.

The following equation is used to estimate the minimum number of cDNA clones required for a complete cDNA library.

$$N = \frac{\ln(1-P)}{\ln(1-1/n)}$$

Where P is the probability, n is the fraction of the mRNA species under consideration and N is the minimum number of the cDNA clones required in the library. For practical consideration, P is usually 99%. Based on this consideration, at least 200,000 recombinant cDNA clones are required to cover all of the mRNA species present in a eukaryotic cell.

III. General Steps of Cloning cDNA Molecules

There are four general steps involved in cloning cDNA molecules: (i) preparation of ds-cDNA molecules, (ii) construction of cDNA-vector chimera, (iii) amplification of cDNA-vector chimera, and (iv) screening and characterization. Depending on the frequency of mRNA under consideration and the availability of probes for detecting the desired cDNA clone, the actual strategy for constructing a library will vary. In this section I will attempt to discuss all these issues.

(1). Preparation of ds-cDNA molecules for cloning

(a). Preparation of glassware and reagents for RNA extraction:

All glassware used in RNA extraction should be washed with detergent and baked at 150°C for at least 6 hr. All plasticware can be treated with 0.1% diethylpyrocarbonate (DEPC) or 10% H₂O₂. Reagents that do not react with DEPC can be made RNase free by treatment with DEPC (made 0.1% final concentration) and followed by autoclaving to remove the excess amount of DEPC. RNase free water can be prepared by the same method.

DEPC cannot be used as an RNase inhibitor for those solutions whose active components are reactive to DEPC. In these cases, the reagents are prepared in RNase free water.

(b). Preparation of total and poly(A)⁺-RNA:

Complete control of RNase activity during the course of RNA extraction is the key to the successful isolation of undegraded RNA from any source. In this regard, vanadyl ribonucleoside complex (VRC) has been shown to be an efficient RNase inhibitor. Therefore, VRC is routinely incorporated in the phenol-chloroform extraction method. Other potent RNase inhibitors are guanidine thiocyanate and guanidine hydrochloride because these two compounds are strong denaturation agents. Methods have also been developed for RNA extraction by the use of guanidine thiocyanate and guanidine hydrochloride buffer. In both methods, the ratio of tissue to extraction is very important. For the liver tissue, 1-2 g of tissue is routinely homogenized in 10 ml of the extraction buffer.

Another important factor leading to the success of RNA extraction is deproteinization. This is commonly achieved by repeat extraction of the tissue homogenate with phenol, phenol-chloroform and chloroform,

respectively. The RNA is recovered by precipitation with 2.5 volumes of ice-cold ethyl alcohol.

There are several different methods developed for extraction of undegraded RNA from different types of tissues based on the above consideration. Detailed protocols for preparation of undegraded RNA at mini and maxi scales will be discussed in lectures.

Poly(A)⁺-RNA is isolated from total RNA by affinity column chromatography on oligo-(dT) cellulose. For routine reverse transcription purposes, RNA isolated by one pass through an oligo-(dT) column is required. The detailed protocols for RNA extraction and poly(A)⁺-RNA isolation are described elsewhere in this publication.

(c). Synthesis of 1st and 2nd cDNA:

The first strand cDNA can be synthesized *in vitro* by reverse transcription of poly(A)⁺-RNA, using oligo-(dT) as a primer and catalyzed by reverse transcriptase isolated from avian myelocytomatosis virus. Generally speaking, successful cDNA synthesis depends primarily upon total control of RNase activity during the reaction. To achieve this goal, a potent RNase inhibitor, RNasin, is routinely used. Since ethanol is a potent inhibitor for reverse transcriptase, it should be removed from the RNA samples completely. The standard conditions for the synthesis of the first strand cDNA is summarized below:

Tris-HCl(pH 8.3)	50	mM
MgCl ₂	10	mM
β-mercaptoethanol	40	mM
dXTP-dCTP	1.25	mM
dCTP	0.5	mM (a small amount of α-32[P]-dCTP is included in the reaction in order to estimate amount of cDNA synthesized)
RNasin	30	U
Reverse transcriptase	50	U
Poly(A) ⁺ -RNA	1	ug

Incubate at 42°C for 30 min.

In addition to the integrity and the secondary structure of mRNA, the concentrations of Mg²⁺ ion, reverse transcriptase, and dXTP will affect the length as well as the yield of cDNA synthesized *in vitro*. Therefore, in order to increase the length and the yield of cDNA, it is advisable to vary these conditions sequentially in the course of cDNA synthesis.

The second strand cDNA is synthesized from the RNA-cDNA duplex under the following conditions:

Hepes (pH 7.0)	10	mM
MgCl ₂	5	mM
KCl	100	mM
dXTP	40	mM
<i>E. coli</i> DNA polymerase I	25	U
RNase H	1	U

Incubate at 15°C for 2 hrs.

(2). Preparation of Chimeric Vectors

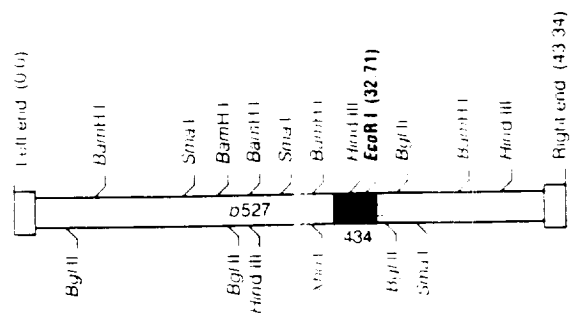
The second step in constructing a cDNA library is to ligate all of the ds-cDNA molecules synthesized into an appropriate cloning vector for amplification in *E. coli* cells. As mentioned earlier, the minimum number of the cDNA inserts to be amplified should be more than 200,000 in order to cover the entire mRNA population. Traditionally, plasmid has been used as a cloning vector for amplified DNA molecules in *E. coli* cells. However, due to low transformation efficiency, plasmids are not suitable cloning vectors for constructing a cDNA library.

In recent years, several lambda phage insertional cloning vectors have been developed. Due to their high efficiency *in vitro* packaging of chimeric phage DNA into infectious phage particles, these vectors are the vector of choice for constructing a cDNA library. Examples of such cloning vectors are lambda gt 10, lambda gt 11, and lambda Zap II (Fig. 2).

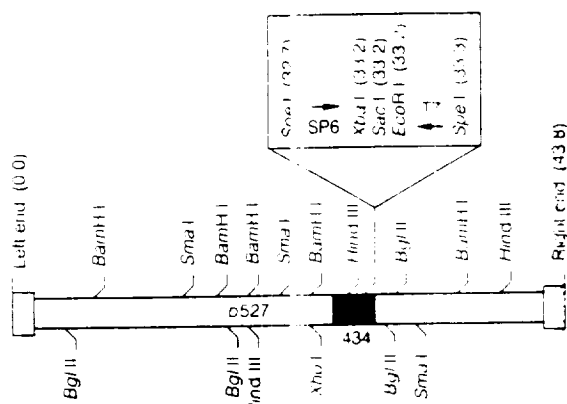
Double-stranded cDNA molecules can be ligated to cloning vectors via the following methods: (i) homopolymer tailing (dG-dC or dA-dT oligomer), (ii) linker ligation (single or double linkers), and (iii) adaptor ligation. Figs. 3-5 are examples of ligating ds-cDNA molecules to cloning vectors.

(3). Amplification and Screening

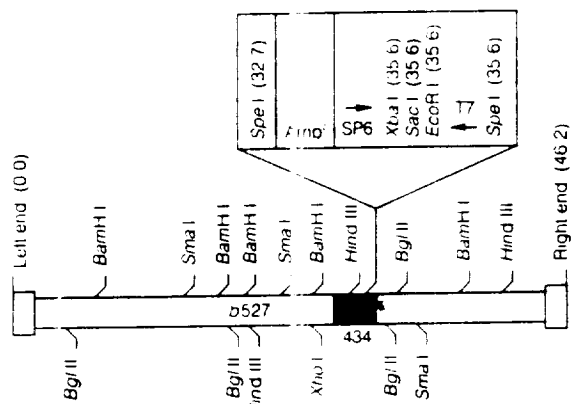
Chimeric DNA is packaged into infectious phage particles by *in vitro* packaging reaction. It is important to remember that at least 200,000 phage particles are required for any cDNA library. However, in order to be sure that more than one desired cDNA clone can be isolated in each screening, it is a good idea to package up to 1×10^6 phage particles per cDNA library.



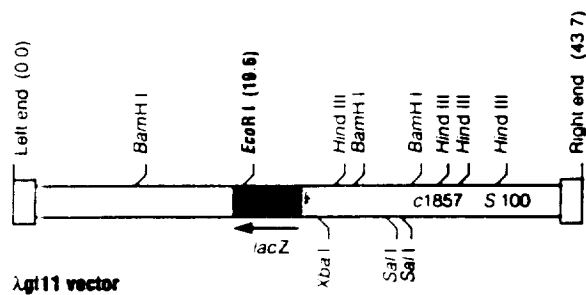
λgt10 vector



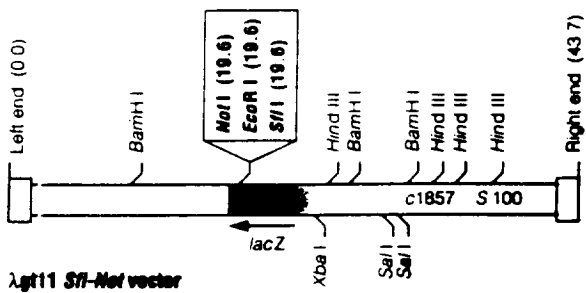
λGEM-2 vector



λGEM-4 vector



λgt11 vector



λgt11 S71-Not vector

Fig.2a

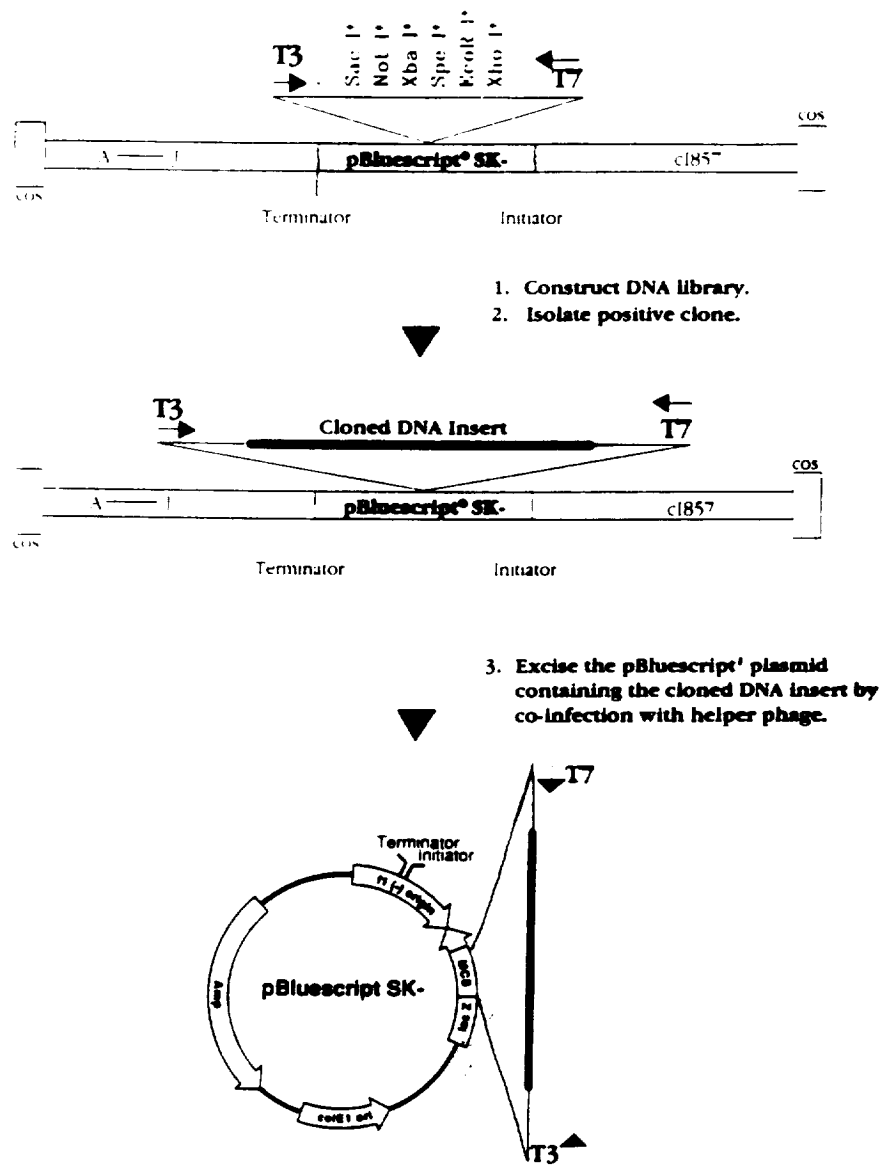


Fig.2b

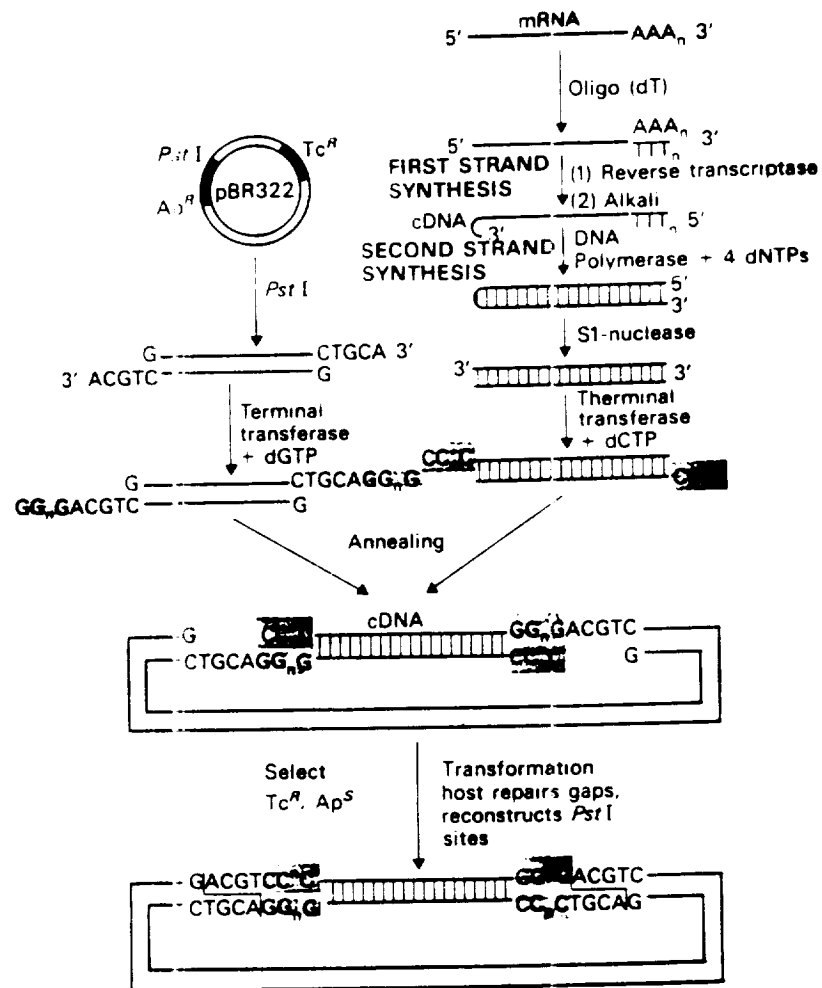
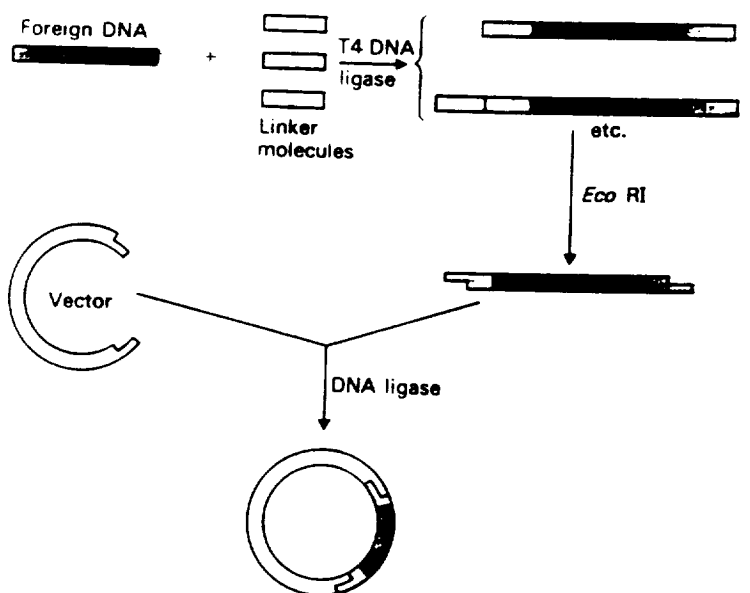


Fig. 3



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Diagram Showing Random Orientation Cloning (left) and Orientation Specific Cloning (right)

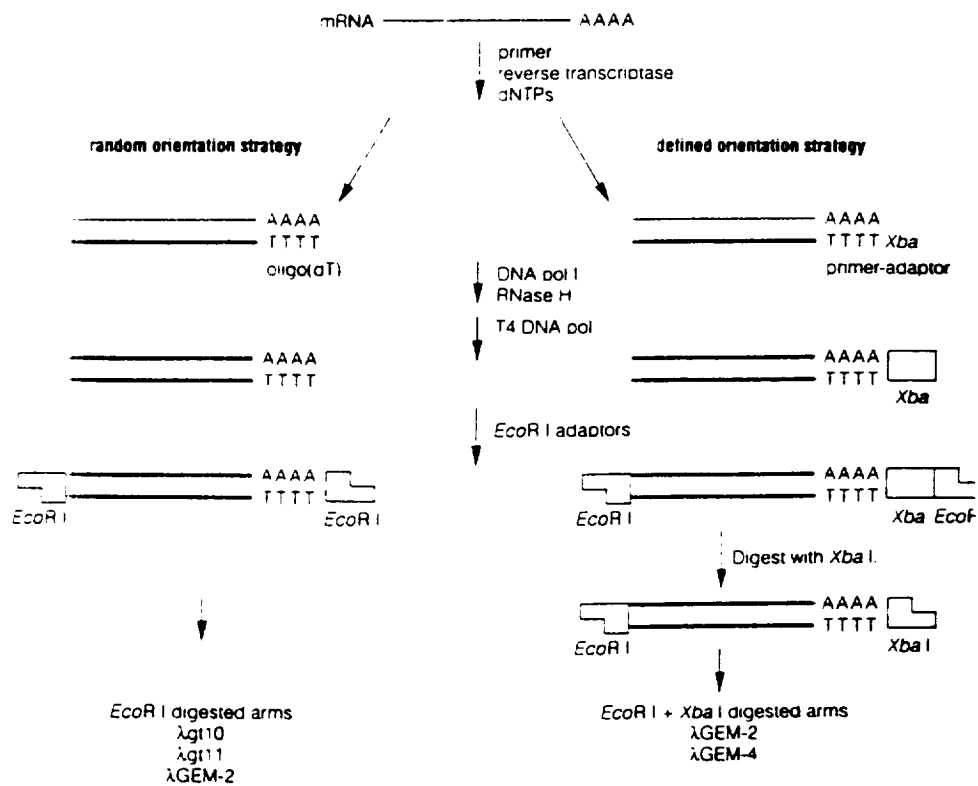
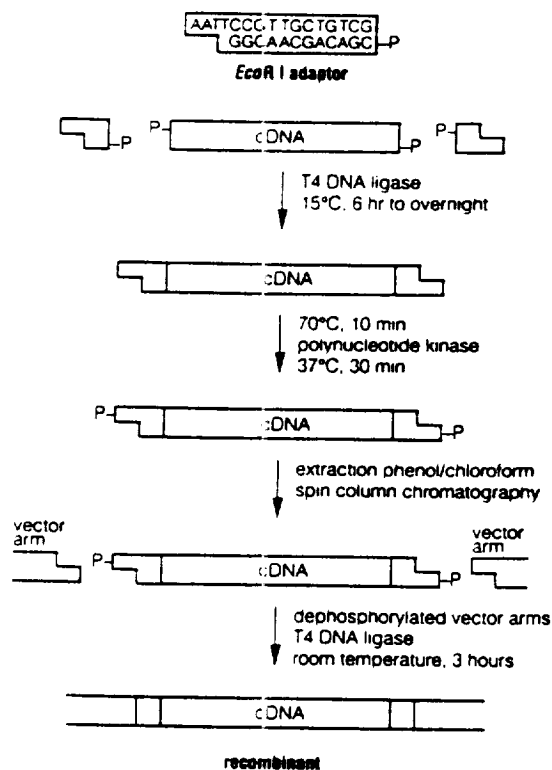


Fig.5



Schematic Representation of *EcoR*I Adaptor Ligation System

Before a library is screened for any desired cDNA clone, it is routinely amplified once by infecting every phage particle to *E. coli* cells. It is important to note that every library can be amplified only once or the slow growing recombinant phage particles will be eliminated in the subsequent round of amplification.

Depending on the availability of probes, a cDNA clone can be isolated from the cDNA library by any of the following three methods:

- (i) Nucleic acid hybridization with a cDNA, genomic DNA, or oligonucleotide probe.
- (ii) Immunochemical screening with an antibody probe.
- (iii) Screening based on the biological activity of the protein encoded by the cDNA clone.

(Examples of each screening method were discussed in detail during the lectures.)

IV. Various Basic Strategies of Cloning cDNA Molecules

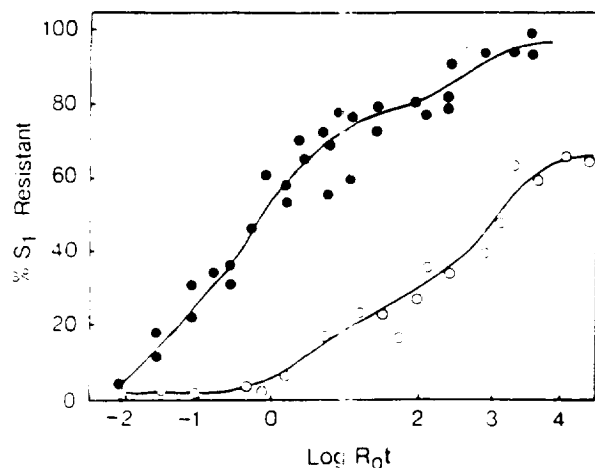
There are two basic factors that determine a particular strategy to be adopted for cloning a cDNA sequence. These two factors are:

- (i). The kind of probe available for the isolation of a cDNA clone of interest from a cDNA library.
- (ii). The relative abundance of an mRNA species of interest.

These two factors will dictate the actual cloning strategy that one should follow in order to isolate the cDNA sequence of interest. In the following section, several examples of cDNA cloning strategies will be discussed.

(1). Strategy of cloning a cDNA sequence when its mRNA is present under a particular condition

In the event that you do not have a known nucleic acid or antibody probe for the cDNA sequence that you intend to clone, you can still isolate such a cDNA sequence if the corresponding mRNA can be induced by a specific hormone, or if it is present in a specific cell type or a specific developmental stage. There are several examples like this in the literature.



The effect of estradiol treatment on mRNA sequence complexity in the male trout liver. [^3H]cDNA transcribed from the total liver RNA of estradiol-treated male fish was hybridized with the total liver RNA of control and hormone-treated fish at 68°C to the R_0t values indicated. The extent of hybridization was determined by S_1 nuclease digestion of the reaction mixture. ●, total cDNA to total liver RNA of the estradiol-treated male fish; ○, total cDNA to total liver RNA of the control male fish.



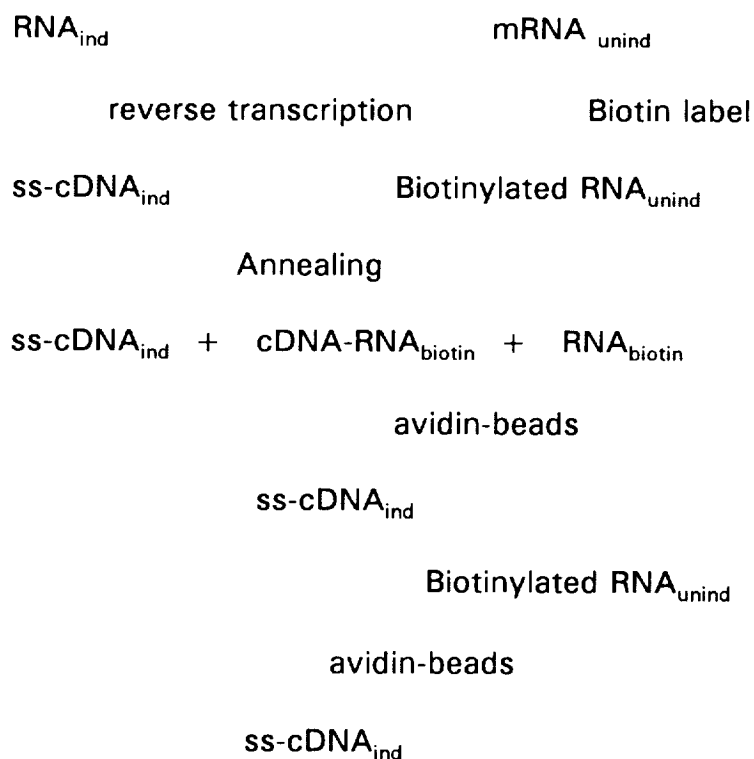
a b

Northern blot hybridization of trout liver RNA with cDNA. Total liver RNA of control and estradiol-treated male fish were electrophoresed on agarose gels in the presence of methylmercuric hydroxide and then transferred to nitrocellulose filter. ^{32}P -labelled cDNA (sp.act., 1×10^8 cpm/ μg DNA) was prepared by reverse transcription using total liver RNA of hormone-treated male fish as a template. The hybridization of cDNA to high frequency class hormone-specific mRNA was detected by exposure of the x-ray film to the filter for 12 h at -70°C . (a) Twenty micrograms of total liver RNA of control male fish; (b) 20 μg of total liver RNA of estradiol-treated male fish.

Fig.6

Case I. This example shows the isolation of estrogen-inducible cDNA sequences (VG and ULER2 mRNA) in the liver of rainbow trout (Chen, T.T. et al. *Physiol. Zool.* **62**:25-37, 1989). Upon Rot and northern RNA blot analyses (Fig. 6), it is obvious that two mRNAs (i.e., VG and ULER2) are induced by estrogen in the livers of male and female rainbow trout. Since these two mRNAs are present in very high abundance, their cDNAs can be cloned by the following simple strategy.

- (i). Estrogen-induced RNA ----> ds-cDNA----> insert into lambda gt10 or lambda Zap II II-----> cDNA library
- (ii). Screen the cDNA library for VG and ULER2 cDNAs using nucleic acid probes prepared by the following methods.
 - (a). Prepare high specific activity ss-cDNA by reverse transcription using estrogen-induced RNA as templates. This cDNA can be used as a probe in plaque hybridization with the presence of 100-fold excess of uninduced liver RNA for the isolation of VG and ULER2 cDNAs.
 - (b). Prepare enriched estrogen-specific cDNA probes by the following method. This enriched cDNA preparation can be used as nucleic acid probes for the isolation of VG and ULER2 cDNA by plaque hybridization.



The above ss-cDNA is highly enriched with estrogen-responsive cDNA sequence, and hence can be used as a probe for the isolation of estrogen-induced cDNA sequences after it is radio-labelled by kinase reaction. Alternatively, this ss-cDNA can be used as templates for the construction of a cDNA library by strategies to be discussed in the next section.

Case II. If the mRNA in **Case I** is present in trace quantity, the following strategy can be followed.

In this case, the enriched estrogen-responsive cDNA can be amplified by PCR and then cloned into an appropriate cloning vector, e.g., lambda Zap II. The cloning strategy is described below:

-----TTTTT5'

Terminal transferase to add "Cs"

CCCCC-----TTTTT5'

use oligo(dG)-R1 and oligo(dT)-R1 as
primers in PCR to amplify the entire cDNA
population

R1-CCCC-----TTTTT-R1
R1-GGGG-----AAAAA-R1

Methylation,
Digestion with restriction enzyme EcoR1,
Pass through Sephacryl 300 column

Ligate to lambda gt10 or lambda Zap II
in vitro packaging and amplification in
E. coli cells

A PARTIAL cDNA LIBRARY

Screen the library with the enriched
estrogen-responsive cDNA as a probe

CLONES-----ready for further characterization

Note: These two strategies are very useful in cloning cDNA sequences whose corresponding mRNAs are present under one condition and absent in another condition. Examples are tissue specific mRNA, developmental stage specific mRNA, and hormone or other agent inducible mRNA.

2. Strategy for cloning a cDNA sequence when portions of the corresponding peptide sequence are known

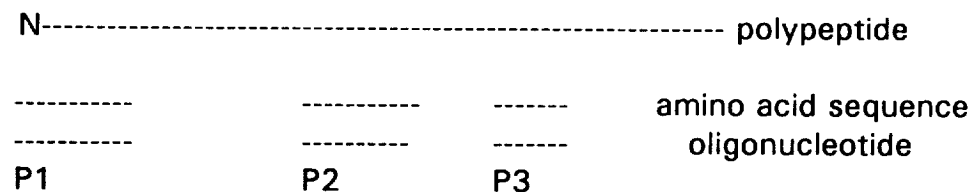
(i). If the amino acid sequences of several portions of a polypeptide are known, their corresponding nucleotide sequences can be derived by referring to the genetic codes. Synthetic oligonucleotides can be made from this information and used as probes for the isolation of the corresponding cDNA.

example:

peptide:	Cys	Ilu	Met	Ala	Glu	Gly	His	Arg	Asp	Cys
Nucleotide	UGU	AUU	ATG	GCN	GAA	GGN	CAU	CGN	GAU	UGU
	C	C			G			C C	A	C C
		A							G	

A mixed oligonucleotide of any length (17 to 27 mer) can be synthesized based on the sequence shown above. One point worth noting is that the length of the oligomer should be long enough to preserve the specificity. However, it can not be too long or the sequence combination will be too high, and consequently the concentration of the correct oligomer will be too low. More details about the design and use of the oligomer were discussed in lecture.

(ii). If several oligonucleotides can be derived from different portions of the polypeptide, these oligonucleotides can be used as primers for direct PCR amplification of a portion of the cDNA sequence. After characterization, the amplified cDNA fragment can be used as a probe for the isolation of the full-length cDNA from a cDNA library. The strategy of the PCR amplification is described below:



Strategy of PCR amplification:

Poly (A)⁺-RNA

reverse transcription

----- cDNA

P1 and P3 as primers

PCR amplification

Blunt end the amplified DNA molecules

Clone into plasmid or M13

Isolate positive clones by hybridization with P2

Positive Clones

confirm the result by nucleotide sequence

determination

Clones are ready to serve as a probe
for isolation of the desired cDNA clone.

(iii). Assume you have some amino acid sequence information on the cDNA that you are interested in, but the amount of tissue that you can use for RNA extraction is very limited. Obviously you can not prepare a conventional cDNA library for the isolation of the desired cDNA clone. In this case, the following strategy can be tried. This is called a RACE method to prepare a cDNA library. The strategy is outlined below:

N-----	polypeptide
-----	amino acid sequence
R1-----	-----R1 oligonucleotide

Poly(A)⁺-RNA

reverse transcription

-----TTTTT5'

addition of poly(G) by terminal transferase

3'-----TTTTT5'

R1-oligo(C) and P2
as primer, PCR

R1-oligo(T) and P1
as primer, PCR

Digestion with restriction
enzyme EcoR1
Ligation of the amplified
DNA to lambda Zap II
in vitro packaging and amplification

A Partial cDNA Library

A Partial cDNA Library

Select cDNA clones
using P1 as a probe

Select cDNA clones
using P2 as a probe

cDNA Clones(with 5' end)

cDNA Clones (with 3')

By sequencing both cDNA clones, the entire cDNA sequence can be deduced. The detailed procedure of the RACE method is described in Appendix I.

(3). Strategy of cloning cDNA sequence that can be isolated by immunochemical reaction.

If a monospecific polyclonal antiserum against a particular polypeptide is available, the corresponding cDNA clone may be isolated by cloning the desired cDNA into a cloning vector under the regulation of a prokaryotic promoter so that the cloned cDNA sequence can be expressed into its gene product. These cDNA clones can be easily screened by immunochemical screening method. Both lambda gt11 and lambda Zap II are such cloning vectors.

There are several situations when this technology is particularly useful, including: (i) existing knowledge is at the protein level (both biochemical characteristics and bioassay); (ii) screening with a nucleic acid probe has failed; and (iii) heterologous antibodies can also be used as powerful probes.

However, there are a few limitations about this approach. These are:

- (i). The protein must be purified or isolated
- (ii). A large (1-2mg) amount of protein must be available
- (iii). Some proteins are not suitable
- (iv). Screening can be relatively tedious
- (v). Success relies strongly on the nature of an unpredictable immunological response
- (vi). Immune response is slow (months).

More detailed information regarding this technique is provided in Appendix II. If the biological activity of the gene product is known, a bioassay of the gene product can also be developed for the isolation of the corresponding cDNA sequence. Examples will be given in lecture to explain such a screening strategy.

Appendix I: RACE

1. Prepare RNA using a protocol that yields high quality RNA. If micro scale preparation of RNA from small tissues is required, the 4 hr prep (also called acid phenol) or other easily scaled down methods are advisable. In small scale preps, add 2 μ g 5s E. coli ribosomal RNA (NOT yeast tRNA).
2. Dissolve the final RNA pellet in 10 μ l dH₂O if using a small scale prep. For larger preps, only 10 μ g RNA is required. mRNA enrichment is not necessary in this method, and in the micro scale preps it is also unadvisable since substantial loss is possible.
3. USE RNase FREE PRACTICES UNTIL NOTIFIED OTHERWISE
 - a. use gloves
 - b. all reagents should be made in DEPC treated dH₂O
 - c. pipet tips and all tubes used should be autoclaved before use
4. First strand reaction
 - a. Add:
 - 10 μ l RNA
 - 2 μ l oligo-dT₁₂₋₁₈ (2 μ g)
 - b. Heat to 65°C for 10 min.; 37°C for 5 min.; then chill on ice for 5 min.
 - c. Add in order:
 - 6 μ l 5X RT buffer
 - 2 μ l 600 mM β -ME
 - 3 μ l 10X dXTP
 - 2 μ l α -P³²-dCTP
 - 30 U RNasin
 - 50 U Reverse transcriptase
 - add water to a final volume of 30 μ l.
 - d. Incubate at 42°C 1 hr., then 52°C for 30 min.
5. Add 0.70 μ l of 1X TE.
6. Heat the reaction to 99°C for 5 min.
7. Spin cDNA through a Sphacryl-300 column, and precipitate the cDNA with 2.5 volumes of EtOH (add 5 μ g 5S rRNA as the carrier). Incubate the mixture at -80°C for one hour. Spin down the pp't in an Eppendorf centrifuge at the top speed for 30 min in the cold. Wash the pp't in 70% EtOH, dry and resuspend in 12 μ l of water. Take one μ l for radioactivity determination (in order to calculate the amount of cDNA made). Take another μ l for size determination by electrophoresis on 1.5% agarose gels.

8. Tailing reaction

a. Add:

- 10 μ l cDNA
- 1 μ l 4 mM dGTP
- 4 μ l 5X tailing buffer (supplied with the terminal transferase)
- 2 μ l terminal transferase (30-60U)
- 3 μ l CoCl_2 (not required with some enzymes, supplied with enzyme if required. Substitute dH_2O if not required)

b. Incubate the reaction at 37°C for 30 min.

c. Heat the reaction mixture at 65°C for 5 min.

d. Precipitate the cDNA by adding 2.5 volumes of EtOH, collect the pp't, wash in 70% EtOH, dry, and dissolve the cDNA in 20 μ l of water.

AT THIS POINT YOU NO LONGER NEED TO MAINTAIN AN RNase FREE ENVIRONMENT

9. PCR

a. Add:

- 10 μ l 10X TAQ polymerase buffer (supplied with the enzyme)
- 2 μ l each of the dNTP (usually at 10 mM stock conc.)
- 1 μ l primer 1 (1 μ g/ μ l)
- 1 μ l primer 2 (1 μ g/ μ l)

b. Add entire cDNA volume

c. Add enough dH_2O so that the reaction volume is now 99.5 μ l

d. Heat at 95°C for 5 min.; cool to 72°C

e. Add 0.5 μ l TAQ polymerase (2.5 U)

f. Add mineral oil

g. Cycle 95°C 1 min.; 55°C 1 min.; 72°C 3 min. for 40 cycles

h. This is a high stringency guideline, actual conditions may be empirically determined

i. Extend at 72°C for 15 min.

11. Remove the mineral oil

12. Digest PCR product with EcoR1

a. digest setup

- 89 μ l PCR product
- 10 μ l 10X EcoR1 buffer (supplied with enzyme)
- 1 μ l EcoR1 (10 U)

b. incubate at 37°C for 1 hr, stop digest with 5 μ l .5 M EDTA and heat to 65°C for 10 min.

c. Run the PCR product digest over a spin column (Sephacryl-300)

14. Quantitate spin column effluent with spectrophotometry or fluorometry
15. Resuspend aliquote of the PCR product to 50 ng/ μ l
16. The ligation
 - a. If a low amount of cDNA is present add:
 - 2.5 μ l DNA (the entire PCR product)
 - 0.5 μ l 10 mM ATP (store on ice < 20 min. or at -20°C)
 - 0.5 μ l 10X ligation buffer (store on ice)
 - 0.5 M TRIS pH 8.0
 - 70 mM MgCl₂
 - 1.0 μ l phage arms(1 μ g) (store ONLY at -20°C)
 - 0.5 μ l HIGH CONCENTRATION LIGASE (store ONLY at -20°C)
 - b. If a high amount of cDNA is present set up 2 reactions

	A	B
DNA	1 μ l (50 ng)	2.5 μ l (125 ng)
10 mM ATP	0.5	0.5
10X lig. buf.	0.5	0.5
arms	1.0	1.0
dH ₂ O	1.5	0.0
HIGH CONCENTRATION LIGASE	0.5	0.5

- c. Mix ligations GENTLY but sufficiently to homogenize
- d. Spin 5 sec. in the microfuge
- e. Ligate 4°C overnight

Comments about RACE

1. With RACE, only one area of nucleotide homology is required.
2. The area of homology can either be 3' within the gene (as in this protocol) or 5', as would be obtained by N terminal amino acid sequencing.
3. If a 5' region is used, the poly A tail of mRNA is used rather than adding a poly G tail.
4. Both PCR primers MUST have restriction sites built onto them, so that they will be clonable into the phage vector.

Appendix II: Screening a cDNA Clone by Immunochemical Methods

I. Raising the antibody:

A. Purification of the protein

1. Traditional chromatographic techniques
2. Resolution on SDS-PAGE

B. Immunization

1. Injection of protein into a rabbit or mouse host
 2. Injection of an acrylamide block containing the protein
 - a. Protein can be reduced or otherwise treated to resolve subunits
 - b. Can aid a difficult purification
 - c. Protein may be more similar to that eventually expressed in the library
 3. The host mounts an immune response to the injected protein in about one month
 - a. Rabbits, mice, rats, sheep, guinea pigs, goats, horses can be used, depending on the volume required
 4. The host is bled before, during, and after immunization
- C. Test the preimmune and postimmune sera by western blot analysis

1. Specificity

- a. To the protein or subunit of interest but not to *E.coli* or lambda

2. Dilution factor (1:200 to 1:10,000)

II. Construction of an expression library

A. cDNA generation

1. "Typical" method

- a. cDNA has two EcoR1 ends
- b. cDNA can be cloned in forward or reverse

2. Asymmetric linking

- a. Two different restriction site ends are added
- b. cDNA only cloned in the forward orientation

B. Choice of vector

1. MUST be an expression vector

- a. Contain an inducible gene with a cloning site within it
- b. Contain the genetics for transcription and translation of the inducible gene and the recombinant insert
- c. lambda gt11 or lambda ZAP II

2. *E. coli* host should have lon-phenotype

- a. Allows excess protein buildup
- b. Y1090 or XL-1

3. *E. coli* host should have lac Z (or other gene) deletion

III. Basic Screening Procedures

A. Preparation of blots

1. Grow infected bacteria 4 hrs. at 42°C
2. Soak NITROCELLULOSE filters in 10 mM
3. Overlay plates with filters
4. Grow 4 hrs to overnight at 37°C or room temperature
5. Expressed proteins adhere to nitrocellulose when filters are lifted off the plates

B. Incubation with the primary antiserum

1. Reabsorb the primary antiserum with *E. coli* extract to render specific to the antigen of interest
2. Filters are first blocked with 1%BSA, 20% BCS, or milk
3. Primary antisera incubation is 30 min at room temperature or overnight at 4°C

C. Incubation with the secondary antibody

1. Secondary antisera is goat anti-rabbit fused to alkaline phosphatase or horseradish peroxidase
2. Filters are incubated with the secondary antibody (1-1000 dil.) for 30 to 60 min at room temperature
3. Wash the filters with blocking solution

D. Color development

1. Alkaline phosphatase or horseradish peroxidase activity is assayed for any phage plaque. The correctly expressed fusion protein will have the rabbit primary antibody bound to the nitrocellulose in a corresponding position.

Appendix III: Amplification of the Lambda Zap-II Library

It is usually desirable to amplify libraries constructed in lambda vectors in order to obtain a large quantity of high titer and stable stock. However, more than one round of amplification is not recommended since slow growing recombinant clones may be significantly reduced in the process of amplification. In some cases, the cDNA library is screened without prior amplification. The host cell strain used to amplify or plate lambda Zap-II clones is *E. coli* XL-blue, which grows well in L-broth.

1. Preparation of Host Cells

E. coli XL-blue strain is used as host cells for the amplification or plating out of the library for screening. The plating host cells should be started from a single colony. Bacterial cells from a single colony are inoculated to 100 ml of L broth supplemented with 0.2% maltose and 10 mM MgSO₄, and grown overnight at 30°C under vigorous shaking. Cells are collected by centrifugation at approximately 6,000 rpm and resuspended in 0.5 volumes in 10 mM MgSO₄. These cells are called plating cells, which are good for up to two weeks at 4°C.

2. Amplification

- (a). Dilute plating cells with 10 mM MgSO₄ so that 600 μ l of the diluted cells will give O.D.₆₀₀ = 0.5.
- (b). Mix aliquots of the packaged lambda clones (about 50,000 recombinant clones) with 600 μ l of the diluted plating cells in Falcon tubes.
- (c). Incubate tubes containing phage and host cells for 15 min at 37°C.
- (d). Mix 8 ml of melted (48°C) top agar (NZCYM broth containing 0.7% agarose) with each aliquot of infected bacteria and pour evenly onto the 150 mm NZCYM plate (NZCYM broth containing 1.5% agar).
- (e). Incubate plates at 37°C for 5-8 hrs. Do not allow the plaques to get too large.
- (f). Flood each plate with 10 ml of SM buffer (containing 5.8 g of NaCl, 2.0 g of MgSO₄, 50 ml of 1 M Tris-HCl (pH 7.5), and 5 ml of 2% gelatin). Incubate the plates at 4°C overnight with gentle shaking or rocking.
- (g). Recover the bacteriophage suspension from each plate and pool into a sterile tube. Add chloroform to 5% and invert to mix.

- (h). Remove cell debris by centrifugation for 5 min at 6,000 rpm.
- (i). Collect the supernatant, pool and store in a glass or polypropylene bottle. Add chloroform to 0.3% and store in aliquots at 4°C.
- (j). Determine the titer of the library.

Appendix IV: EXTRACTION OF HIGH MOLECULAR WEIGHT DNA

- (a). Resuspend 2 ml of red blood cell pellet in 5 ml 1X SSC.
- (b). Add 5 ml of TNE buffer (100 mM Tris-HCl, pH 7.5; 100 mM NaCl; 10 mM EDTA; 1% sarkosyl), and mix gently by rotating the tube for 2-3 min to lyse the cells. (DO NOT SHAKE THE TUBE OR YOUR DNA WILL BE SHEARED INTO PIECES). Add freshly prepared proteinase K to 100 µg/ml final concentration.
- (c). Incubate the tube at 55°C for 2 hr. Then add 10 ml TNE buffer.
- (d). Extract the sample, one time each, with equal volumes (20 ml) of freshly saturated phenol, phenol-chloroform (1:1 v/v), and then chloroform-isoamyl alcohol (24:1 v/v). The organic phase is separated by centrifugation at 3,500 rpm for 10 min. In each extraction, the aqueous phase is transferred to a clean tube with a pipette whose entire tip is cut off in order to avoid (i) shearing the DNA molecules and (ii) pulling the interphase into the DNA solution.
- (d). Transfer the aqueous phase into a dialysis tube and dialyze the aqueous phase against 4 liters of TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) for 24 hr. with several changes of the buffer.
- (e). Estimate the DNA concentration in each sample by electrophoresis of DNA samples on a 0.4% agarose gel (mini gel).

Molecular Approaches to the Analysis of Population Polymorphisms and Enzyme Expression

**Prepared by: Douglas Crawford, University of Chicago
and Simona Bartl, Hopkins Marine Station, Stanford Univ.**

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RNA ISOLATION

Chaos Buffer for RNA isolation

		For 100 mls:
4.5 M	Guanidinium thiocyanate	53.2 g
2%	N-lauroylsarcosine (sarcosyl)	2.0 g
50 mM	EDTA	10 mls of 0.5 M
25 mM	Tris-HCl, pH 7.5	2.5 mls of 1 M
0.1 M	β -mercaptoethanol	700 μ l
0.2%		antifoam A (Sigma)

5.7 M CsCl cushion

	FINAL VOLUME	
		100 ml
5.7 M	"Baked" CsCl	95.8 g
0.1 M	EDTA	20 mls of 0.5 M

Use DEPC treated water and baked glassware. This solution, absolutely, positively must be RNase free. RNase is everywhere! Wear gloves and use only baked glassware or virgin plastic. DEPC treat your water and buffers (except Tris). Be very careful.

- 1 Weigh animal.
Remove organ (liver) and weigh. (One of the superior attributes of cDNA is you reduce the gene complexity by choosing an organ that expresses only a single locus of a multilocus enzyme.)
- 2 Homogenize tissue rapidly in "Chaos" buffer (9 mls).
- 3 Spin homogenant at 10 KG to remove insoluble material.
- 4 While homogenant is spinning:
- 4A Weigh out 1.8 g of CsCl (0.2g/ml) per sample.
- 4B In the ultra-centrifuge tube ("quick-seal") add 3.5 mls of 5.7 M CsCl "cushion." This layer must be RNase free. You will centrifuge your RNA through this layer and any contamination will cause significant loss.
- 5 Remove supernant, put into fresh tube, and add 1.8 g CsCl.
- 6 Carefully add homogenant on top of the CsCl cushion. This is most readily accomplished by using a 10cc syringe and long needle. Place needle/syringe into quick-seal tube, and add tissue homogenant to the syringe. Homogenant will slowly trickle into Q-S tube, floating on top of the cushion.
- 7 Carefully balance matched pairs of centrifuge tube (+/-0.005 g).
- 8 Seal tubes, place matched tubes opposite of each other, and cap with red aluminum tops.
- 9 Centrifuge 50,000 rpms, 5.5 hrs, 20°C.

After centrifugation

Mark tubes where pellet should be: on the bottom outer side (relative to center of rotor)

- 10 Remove tubes, place in convenient rack.
- 11 Slice off the top of tube.
Aspirate off the top 2/3-3/4 of solution. Work from the top down. It is important that you aspirate off the upper-most solution first and remove all but the clear cushion. Do not aspirate the invisible pellet at the bottom.
- 12 Cut off the top 2/3 of tube. Using a "pulled" Pasteur pipet, carefully remove remaining solution. The pellet will be on the bottom-side of the tube.
- 13 Rinse pellet with 70% EtOH, remove (use Pasteur pipets), repeat twice (total 3 washes).
- 14 Add 200 μ l of 0.1xTE (RNase free), using the pipet tip to crush pellet and "titrating" TE in an attempt to put pellet into solution. Form a heterogeneous solution and put into a fresh microfuge tube.
- 15 Repeat with 200 μ l of TE, pooling both solutions.
- 16 Vortex. (Good RNA does not like to go into solution. Patience is a virtue.)
- 17 Determine concentration, 10 μ l into 490 μ l (500 μ l final vol),
- 18 Remove 1 to 2 μ g of RNA for gel analysis (add RNA loading buffers),
- 19 Add 1/10 volume RNase free 3 M NaOAc (40 μ l), 2.5 volumes of EtOH (1.0 ml). Mix vigorously.
- 20 You can calculate the concentration of RNA in the EtOH.
Thus, there is no need to precipitate all of your RNA at once. Just remove about 1.5 to 2 times the amount you need by vortexing EtOH/RNA solution and remove the appropriate volume. This EtOH/RNA solution, stored at -20°C or lower, is stable for years.

While RNA is centrifugating,

- 1 Digest pSP6LDHB with BamHI, so we can use this linearized DNA to produce cRNA.
- 2 Pour RNA gel.

RESTRICTION ENZYME DIGEST

10 g of pSP6LDHB
5 μ l Restriction buffer (medium)
34 μ l dH₂O
1 μ l Bam HI
37°C for 2 + hrs.

Normal DNA digest with restriction enzymes is in a total volume of 50 μ l (microliters, 10⁻⁶ liters, or λ). You can do the digest in a smaller volume if, for example, you are only going to run it on a "minigel." In general you digest 0.25 to 2 μ g (micrograms, 10⁻⁶ grams, γ) per digest. Why 0.25 to 2 μ g? Because you can visualize approximately .05 μ g (50 ng, 50 nanograms) per band on a gel -- this depends on the length of the DNA (the longer the piece (band) the easier it is to see).

Recipe			
"X"	μ l	DNA	.25 to 1
5	μ l	10x buffer	Use the correct buffer (see chart)
49	μ l - "X" μ l	dH ₂ O	Final volume equals 50 μ l
1	μ l	ENZYME	1 μ l of restriction enzyme always is greater than 1 unit. It is usually 10 to 20 units. What's a unit? 1 unit is enough enzyme to digest 1 μ g DNA. Thus, even if you were to digest 10 μ g of DNA you would have enough enzyme. Enzymes are expensive.

Incubate 37°C for 1 to 3 hours.

Sometimes you need to add Mg⁺⁺ to the reaction (in the form of MgCl) because you add a large volume of DNA. That is, if "X" is large (> 20 λ) you need to add Mg. Why? DNA is usually stored in a buffer with EDTA. EDTA binds 4 moles of Mg for every mole of EDTA. Thus, DNA in a 1 mM (millimolar) solution of EDTA will bind 4 mM of Mg. A good rule of thumb is for every 20 λ of DNA add 1 λ of 10 mM MgCl.

Note: Enzymes should always be kept cold. Always keep them on ice and minimize the time you have them out of the freezer.

***IN VITRO*-PRODUCED MRNA AND CRNA**

ALL REAGENTS MUST BE RNase FREE!!!!

SP6 RNA Polymerase 10X buffer

		for 1ml
400	mM Tris-HCl pH 7.5	400 μ l
60	mM MgCl ₂	100 μ l of 1 M
20	mM Spermidine	20 μ l of 1 M
100	mM NaCl	20 μ l of 5 M
100	mM DTT	400 μ l of 1 M

AT ROOM TEMPERATURE, ADD IN ORDER

10 λ 5x Transcription Buffer
5 λ 100 mM DTT
5 λ 10 mM rNTP (10 mM @ of rATP, rCTP, rGTP, rUTP)
2-5 γ Linearized plasmid
30-50 units of SP6 or T7 RNA polymerase

final volume 50 λ , DEPC dH₂O
90 minutes, 37°C .

Add 1 unit "RNase free" DNase 1 u/ γ
20 min 37°C ,
50 μ l 10x TE
100 λ phenol, vortex, 68°C 5 min. vortex
spin, remove supernant into new tube,
phenol:chloroform extract
chloroform extract
1/10 vol. 3 M NaOAc, 2.5 vol EtOH

OPTION:

To determine the exact yields, add 25 μ Ci of ³H-UTP. Use ³H-UTP so that when you use cRNA as a positive control for ³²P assay, you can count the cRNA in low energy window and ³²P in a high energy window (i.e., when using a liquid scintillation counter).

RNA GEL

To verify the integrity of your RNA you should do a "*Northern*" analysis. In order to accomplish this you must run a denaturing gel.

		for 100 mls
1.2 %	Agarose	1.2 g
1x	E buffer	2 mls of 50X
	dH ₂ O	82 mls

Heat, put agarose in solutions, let cool to 60°C
add 16 ml of Formaldehyde
pour gel, in hood if possible

Running Buffer, 1 XE, 3% formaldehyde (1/12.5 of vol, because formaldehyde is 37%)

Heat 2 µgs of RNA at 85°C, quick cool
add 2.5 vol of RNA Denaturing Reagent
1/10 vol Loading Dyes

RNA Denaturing Reagents

		for 400 µl
80%	Formamide-deionized	300 µl
3.7%	Formaldehyde	40 µl
1X	50X E buffer	8 µl

NORTHERN BLOT

- (1) Rinse gel with dH₂O.
- (2) Set up capillary blotting (just like a Southern).
- (3) After blotting, air dry.
- (4) Wrap filter in plastic wrap, UV irradiate, 3.5 mins.
- (5) Wash in 2X SSPE 0.1% SDS 10 mins.
- (6) Wash in hot (85-95°C) 2X SSPE, 0.1% SDS, 1 min.
- (7) Pre-hybridize 37°C in 5X SSPE, 50% formamide, 5X Denharts, 100 µg/ml CTDNA, 50 µg/ml yeast RNA.
Minimum of 6 hours pre-hybridization.
- (8) Add labeled probe, hybridize (12- 48 hrs).
- (9) After hybridization, wash filter at 65°C, 2 min, 2X SSPE.
- (10) Second wash 65°C in 2X SSPE, 0.1% SDS, 10 min.
- (11) Third wash, same as the second.
- (12) Fourth wash room temperature in 0.2X SPPE, 0.1% SDS.

FRAGMENT PURIFICATION: AGAROSE

Run DNA on "*Low melt*" agarose gel: 1% -- 600bp to 5kb; 2% -- 300 to 700bp. Smaller? use 2% "*Nusieve*" + 1% "*Low melt*".

After bands have separated, visualize bands on UV box (minimize exposure of DNA to UV), cut bands out (do not scratch filter on box).

Add 100 λ of TE (10 mM Tris-HCl pH 7.6, 1 mM EDTA) to band, crush, heat to 65°C for approx. 5 min, add 200 λ of phenol, vortex, heat 65°C 3 min, vortex.

Microfuge 5 mins, remove supernant.

Add 100 λ of TE to phenol, vortex, heat 65°C 3 min, vortex.

Microfuge, pool supernants.

Chloroform extract (approx. 400 λ), microfuge 3 mins.

EtOH precipitate, 1/10 vol. 3 M NaOAc, 2.5 vol. EtOH.

-20°, 1-2 + hrs.

RANDOM PRIMING

10x K buffer

	for 1 ml
100 mM Tris-HCl pH 7.5	100 μ l of 1 M
100 mM MgCl ₂	100 μ l of 1 M
50 mM DTT	50 μ l of 1 M
dH ₂ O	750 μ l

2 λ 25- 100 ng of DNA Fragment

23 λ dH₂O

95-99°C , 5 mins

Quick cool

add

2.5 λ 10X Klenow buffer

5 λ ³²P- dCTP

2.5 λ 10 mM dA,dG,dT mix

1 λ 90 A unit/ ml Random Primers (hexanucleotides)

1 λ Klenow (Large Fragment of DNA Polymerase)

RT 30 min, 37°C 30 mins

add 50 λ 10X TE

G-50 spin column.

determine volume with pipetman, count 1 λ in LSC.

MRNA TO CDNA

mRNA can be converted into DNA (copy DNA, cDNA) by annealing oligo-dT to the 3' poly-A tail that occurs on all eukaryotic mRNA. After the dTs bind to the As, we will use the enzyme "Reverse Transcriptase" to read the RNA into DNA. This DNA can be used for many purposes; we plan to use it as a substrate for PCR amplification.

5X Reverse Transcriptase Buffer

	for 10 mls
250 mM Tris HCl pH 8.3	2.5 ml of 1M
40 mM MgCl ₂	400 μ l of 1M
150 mM KCl	150 μ l of 1M

X μ l 20 μ g of total RNA (which represents about 0.2 μ g of mRNA)
2 μ l 0.05 μ g of dT₁₅ (oligo dT, which is 15- 18 nucleotides long)
20-X μ l of dH₂O (i.e., total volume equals 20 μ l).
Heat to 90-95°C 5 min.
Quick chill on ice.

To 20 μ l of RNA-dT add:

12 μ l	5x Reverse Trans. Buffer
6 μ l	50 mM DTT
6 μ l	10 mM dNTP (dATP, dCTP, dGTP, dTTP)
26 μ l	DEPC dH ₂ O
mix	
1 μ l	RNase inhibitor (this is a sensitive protein, requires at least 1mM DTT)
1 μ l	Reverse Transcriptase

15 min room temperature,
30 min 37°C.

OPTIONAL:

Before beginning incubation, remove 10 μ l, add 1 μ l of ³²P-dCTP, incubate as above. After incubation, TCA precipitate with 100 μ g of carrier DNA. After incubation add 40 μ l of 10x TE.
Phenol extract/ phenol-chloroform extract;
add 1/10 vol 3M NaOAc, 2.5 vol. EtOH
-20°C for 2-24 hrs.
Spin, bring up in 20 μ l 0.1X TE.

SOUTHERN BLOT

Denaturing Solution:

		for 1 liter
0.5 M	NaOH	50 ml of 10 M
1.5 M	NaCl	87.6 g

Neutralizing Soln.

		for 1 liter
0.5 M	Tris- HCl pH 7.5	500 ml of 1 M
1.5 M	NaCl	87.6 g
0.1M	EDTA	20 ml of 0.5M

20X SSC

		for 1 liter
3M	NaCl	175.3 g
0.15M	Na Citrate	88.2 g
800 mls	dH ₂ O	
pH to 7.0 with HCl (0.1-0.5 mls approx)		

- 1 Photograph gel with ruler.
- 2 Denature gel 30 mins. RT, shaking, with 0.5 M NaOH, 1.5 M NaCl.
- 3 Rinse with H₂O 2x-3x times.
- 4 Neutralize, 30 mins., RT, shaking.
- 5 While neutralizing, cut membrane to fit gel, cut 3MM paper (3-4 sheets) to size of the gel, prepare appropriate size paper towels for blotting.
- 6 Set up blotting,
2 layers of 3MM paper as wicks into 20X SSC
gel on top of wicks, remove any bubbles
Pre-wetted membrane (in 2X SSC) on top of gel
3MM paper on top of membrane
paper towels on top of 3mm paper
500 g weight on to of paper towels



- 7 6-18 hrs to blot.
- 8 Air dry filter, UV irradiate 3.5 mins.
- 9 Wash filter in hot 2X SSC 0.1% SDS twice.

HYBRIDIZATIONS: RNA OR DNA

2X Hybridization soln.

	for 100 ml
10X SSPE	50 ml of 20X
10X Denharts	20 ml of 50X
0.2 % SDS	2 ml of 10%

Denhardt's soln.

	for 100 ml
Ficoll	1 g
polyvinylpyrrolidone	1 g
BSA (fraction V)	1 g

20X SSPE

	for 500 ml
3.6 M NaCl	105.2 g
0.2 M Phosphate pH 7.0	100 ml of 1 M
20 mM EDTA	20 ml of 0.5 M

Phosphate Buffer pH 7.0

	for 500 ml
Na H ₂ PO ₄ (monobasic)	140 ml of 1 M
Na ₂ H PO ₄ (dibasic)	360 ml of 1 M

Hybridization soln. (HB)

Make fresh when needed. Denature DNA by boiling 5 mins

Deionize foramide with mix bed resin (Biorad AG-501-X8)

10 mls of 2x

10 mls of foramide

1 mg of sonicated denatured DNA (calf thymus, salmon sperm)

for Northern

500 µg of clean (Prot. Kase, Phenol) yeast RNA.

Use HB, to pre-hybridize 6 to 12 hrs; less if lots of target DNA.

Add probe directly to HB soln. Hybridize 12 to 36 hours depending on amount of probe, qualitative vs. quantitative results and amount of target.

After hybridization,

Empty hot soln. into waste container, rinse with minimum vol of 2X SSPE 0.1% SDS, twice, putting rinse into waste.

(You want to minimize the amount of hot waste you produce.)

Wash 2X SSPE 0.1% SDS, 65°C 15 min twice

Wash 0.2X SSPE 0.2% SDS RT 15 min

RNA DOT BLOT

- 1 Prewet 2 sheets of 3MM and 1 sheet of nylon membrane, cut to the size of dot blotter.
- 2 Place paper and membrane into dot blotter, membrane on top.
- 3 Low negative pressure add 0.1% solution of India ink to upper right corner.
- 4 Sample prep: heat 2 -10 μ g of total RNA to 65°C, 10 min, quick cool, add 2.5 vol DNA denaturing reagents. Add 1-2 μ l of 1% loading dyes (dyes make it easier to tell if you have loaded a sample successfully). Include some yeast DNA as a negative control.
- 5 cRNA: prepare sample as above using a serial dilution from 200 pg to 2 pg (dilute in a solution of yeast RNA so total nucleic acid is constant).
- 6 Load sample onto blotter, be sure there is not an air lock (use pipetman to remove air).
- 7 After blotting, air dry, wash in 5X SSC 0.5% SDS, RT 10 mins.
- 8 UV irradiate 3.5 mins.
- 9 Wash in hot 2X SSC 0.1% SDS, twice.

Prehybridize 12+ hours, hybridize 24 + hours.

- 10 Wash as before for Northern/southerns.
- 11 Autoradiograph 12 hours.

After Autoradiography,

- 12 Label 3 ml scintillation tubes for samples.
- 13 Add 400 μ l of 0.1 N NaOH to tubes.
- 14 Carefully add equal size cut out dots to tubes, plus some negative controls.
- 15 Incubate 37°C 15 mins, cool, add 2.5 mls of LSC fluid, mix well.
- 16 Let sit for 30 minutes.
- 17 Count for four minutes, twice (repeat counts after all samples have been counted, greater than 1 hour between first and second). Are the counts for each sample the same?

T4 DNA Polymerase

To be sure you have a blunt end (e.g., on PCR product) or to convert 3' and 5' overhangs to blunt ends, use T4 DNA polymerase with 0.5 mM dNTP.

10X T4 buffer

		for 10 ml
0.5 M	Tris-HCl pH 8.0	5 ml of 1 M
50 mM	Mg Cl ₂	500 µl of 1 M
50 mM	DTT	500 µl of 1 M
100 µg/ml	BSA	100 µl of mg/ml

Reaction:

up to 2 µg	DNA
4 µl	10X T4 buffer
2 µl	10 mM dNTP
dH ₂ O up to 39 µl	

1 µl to T4 DNA polymerase
incubate 30 minutes 37°C

Note: read page 3.5.11-12 in "Red Book" for more details

up to 2 µg

Media for Bacteria

LB:

for 1 liter
10 g Tryptone
5 g yeast extract
5 g NaCl
100 μ l 10 N NaOH

Superbroth:

for 1 liter
20 g Tryptone
10 g yeast extract
5 g NaCl
500 μ l 10 N NaOH

Additives:

	final	stock	add
Ampicillin	100 μ g/ml	50 mg/ml	2 μ l/ml
Kanamycin	70 μ g/ml	35 mg/ml	2 μ l/ml
Tetracycline	15 μ g/ml	15 mg/ml	
light sensitive	in 70% EtOH		1 μ l/ml
(Do not use with media containing MG)			
X-Gal	25 μ g/ml	25 mg/ml	1 μ l/ml
IPTG	0.1 mM	100 mM	1 μ l/ml

Plates:

15 g/liter for agar plates.

Add additives only when media cools to approximately 50 to 55°C.

Most all plasmids have AMP resistant genes, and so need to be grown in media with ampicillin.

XL-1 has a special episome with Tet resistance for blue-white selection and for the production of single-stranded DNA; therefore, use tetracycline when growing up XL-1.

When plating a recombinant plasmid (one in which you hope to have cloned a piece of DNA into the polylinker) you will disrupt the β -lac gene and thus the bacteria (DH-5 a, or L1-1) cannot turn X-Gal blue. Therefore, white colonies should be recombinants.

Preservation of Animal Tissue at Room Temperature for DNA Analyses

(from Seutin, et al., *Can. J. Zool.* **69**:82, 1991)

This protocol has worked well for finely minced tissues and cell suspensions from fish and invertebrates.

DMSO-salt solution:

20% DMSO
0.25 M sodium-EDTA
 NaCl to saturation
pH to 7.5 with HCl or NaOH

1. Cut up tissue to rice-size pieces or grind up soft tissues between two frosted slides to make a cell suspension.
2. Immerse in DMSO-salt solution (at approx. 1 g tissue per 3 ml). Leave at room temperature, up to 24 weeks.
3. Remove excess supernatant (tissue and cells will be pelleted by gravity). Grind up in liquid nitrogen. Add DNA lysis buffer and follow DNA isolation protocol.

Geneclean: the Wonders of Glass and DNA

1. Excise band from agarose from gel, estimate vol. and put into labeled microfuge tube. Note: minimize the amount of agarose gel.
2. Add 3 vol. of NaI stock (incubate 5 min. at 50°C). Make sure the gel slice is dissolved.
3. Add 5 μ l of Glassmilk, incubate for 5 min. at room temperature. Glassmilk is a solution of very fine (small) glass particles that bind DNA.
4. Spin solutions for 5 seconds to pellet glass particles with DNA bound to glass. Note: if you spin too long you will not get your DNA back.
5. Remove supernatant, put into separate tube (most likely will not use the supernatant).
6. Resuspend pellet in "New Wash" by vortexing.
7. Spin again for 5 seconds.
8. Remove supernatant.
9. Repeat steps 6-8 two more times.
10. After last wash, remove all of wash solutions. To be sure you remove all of wash solutions, spin for 2 seconds and remove any remaining liquid.
11. To elute DNA from Glassmilk, add 10 μ l of dH₂O, vortex to resuspend, incubate at 55°C for 10 min.
12. Pellet the Glassmilk by microfuging for 30 seconds (you want a firm pellet).
13. Remove supernatant, which has the DNA in it, and put into fresh tube.
14. Repeat steps 11-12 and combine eluent in one tube for a total volume of 20 μ l.

Assume 80% yield when estimating DNA concentrations.

Boiling Minipreps for Plasmid DNA

STET:

8% sucrose	RNase
0.5% Triton X-100	Lysozyme
50 mM EDTA	Isopropanol
50 mM Tris-Cl pH 8.0	TE

1. Take pellet from 1.5 mls of overnight culture and resuspend in 300 μ l STET with 200 μ g of lysozyme. Vortex well.
2. Place on ice for 30 sec. - 10 min.
3. Place in boiling water bath for 1-2 min.
4. Spin for 15-30 min. Pull out pellet with toothpick and throw away.
5. Add 1 volume of isopropanol, leave at -20°C for 15-30 min.
6. Spin for 5 min. Remove supernatant and dry briefly.
7. Resuspend in 50 μ l of TE. Digest 5 μ l with appropriate restriction enzymes and 1 μ l of 10 mg/ml RNase.

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Fluorescent Labeling of Amino-Oligonucleotides

Oligonucleotides that have a free amino group at their 5' ends are readily synthesized on automated DNA synthesis machines, using commercially available amino modifying reagents (Aminolink I and II, Applied Biosystems). These amino-oligonucleotides can then be further labeled with a fluorophore, biotin, or other reagent via the free amino group. The following protocol is useful for labeling oligonucleotides with isothiocyanate (Fluorescein-ITC) or sulfonyl chloride (Texas Red) dyes, for use in in situ hybridizations.

[Editor's Note: Dr. Ed DeLong was an active participant in the laboratory part of our course. He presented lab demonstrations of the use of hybridization probes for the study of environmental biology. In addition, he presented a short lecture outlining some of the uses of these new techniques, and some of the questions that can and cannot be answered using these approaches. The students requested that Dr. DeLong's laboratory protocols, as well as the overhead projections from his lectures, be included in this document. These are in a slightly different format from the lecture abstracts, but they do present in outline form what was discussed in the lecture part of this laboratory session.]

Labeling Reaction

1) Dry 100 μg of gel purified amino-oligonucleotide. Purify the aminolink oligo on a nondenaturing 20% acrylamide preparative gel. An 18mer runs between the xylene cyanol and bromphenol blue. After electrophoresis, locate the oligo by its UV shadow (cast on a fluorescent TLC plate or intensifying screen). Excise the band from the gel and place it in a small vial. Add 1-2 ml sterile dH_2O and elute (with rotation or agitation) overnight. Remove the eluate and filter through a 0.2 μm filter. Measure the A_{260} to quantitate the recovery.

2) Add 200 μl of 50 mM Borate Buffer pH = 9.2 (or 50 mM Carbonate Buffer). Check the pH of the reaction with pH paper. It's important that the pH is greater than 9.0.

Borate Buffer pH = 9.2

Solution A = 0.1 M H_3BO_3

Solution B = 0.1 M NaOH

For 50 mM Borate Buffer: mix 50 ml Solution A and 26.4 ml Solution B. Bring volume to 100 ml with dH_2O and check pH.

- 3) Add 40 μ l of Stock Dye (Stock Dye = 10 mg dye dissolved in 1 ml dimethylformamide). Make dye fresh each time. Molecular Probes (Eugene, Oregon) sells 1 and 10 mg ampules of dye, which are opened, dissolved in DMF, used once, and discarded.
- 4) Place reaction in dark, overnight. In the subsequent handling of the fluor labeled oligonucleotide, avoid excessive exposure to light.
- 5) Prepare Sephadex G-25 by hydrating it in 10 mM Tris HCl, pH=7.5. Let the Sephadex hydrate overnight.

Removing Unincorporated Dye

- 6) Set up one column of Sephadex G-25 for each reaction. (Plastic 10 ml disposable pipettes, plugged with glass wool, work well.) A bed volume of 5-7 ml provides good separation of oligo from unincorporated dye. Collect about 4 drop fractions in a microtiter plate or in epi-tubes. The labeled oligonucleotide is visible as a colored band, and should elute in the void volume, well separated from the intensely fluorescent trailing band, which is the unincorporated fluor.
- 7) Examine the collected fractions under a long wave UV lamp. The first fluorescent peak is the labeled oligonucleotide. Pool these fractions.
- 8) Lyophilize the pooled product in a Speed-vac or the equivalent. Resuspend in 8 M urea, or 80% formamide, to increase the density for gel loading.

Separating the Labeled from Unlabeled Oligonucleotide

- 9) Load the product onto a preparative, 20% (5% x-link) nondenaturing acrylamide gel. A gel thickness of 2 mm is appropriate for purifying the 100 μ g reactions. An oligomer 18 nucleotides in length will run approximately halfway between the xylene cyanol and bromphenol blue in this gel system. Load some xylene cyanol and bromphenol blue into an empty lane to serve as markers. It's a good idea to also run some labeling dye (e.g., FITC, Texas Red) to see where unincorporated dye runs. Electrophorese at 25W constant power for about 4 hours.
- 10) Visualize the unlabeled oligomer by the shadow it casts when it is placed on a fluor-impregnated TLC plate (covered with Saran Wrap) and illuminated with UV light (can also use an intensifying screen as a backing to locate the UV shadow). The labeled product should be slightly above the "shadow" band -- the fluor retards the migration of the oligomer by approximately 1 nucleotide. Excise the fluorescently labeled product, and

place the gel slice in about 1 ml ddH₂O. Allow the product to elute from the gel overnight, preferably with agitation. Remove the eluate, and filter out the acrylamide (0.22 μ m acrodiscs or similar disposable filters work well). Measure the A₂₆₀ and the A_{max} of the fluor (495 nm for fluorescein). A₂₆₀/A_{fluor max} is about 3.0-3.5. Yields are usually about 20-30 μ g of pure, labeled oligonucleotide. Store the purified, labeled oligo frozen at -20°C, in small aliquots of one to a few micrograms.

In Situ Hybridization with **rRNA-Targeted Fluorescent Oligodeoxynucleotides**

MATERIALS

50 ml Falcon tubes
staining jars
slide racks
teflon coated slides (Cell Line Associates, Newfield, NJ)
pipetmen p-20, p-200, p-1000
water bath and dry incubator
coverslips
Citifluor mountant (or glycerol:PBS [9:1])
immersion oil
epifluorescence microscope
Gelatin Subbed Slides:
 Soak clean slides for 2 minutes in a filtered 65°C solution of:
 0.1% gelatin
 0.01% CrK(SO₄)₂ (chrom alum)
 Air dry

REAGENTS

1X phosphate buffered saline (PBS) (pH = 7.6)
methanol
37% formaldehyde (or freshly prepared 8% paraformaldehyde in 1X PBS)
2 M Tris, pH = 7.8
2% BSA (fraction V)
25X SET:
 3.75 M NaCl
 25 mM EDTA
 0.5 M Tris, pH = 7.8
10% SDS
distilled H₂O
rRNA targeted, fluor-labeled oligodeoxynucleotide probes

PROTOCOL

1) Cell Preparation

Centrifuge cell sample, and resuspend in PBS (1/2 X artificial seawater, if of marine origin). Cells can then be fixed by adding 1/10 volume of 37% formaldehyde, followed by storage for 4-16 hours at 4°C. Cells may then be resuspended in 1X PBS buffer and stored at 4°C for up to 8 weeks.

Alternatively, freshly grown or collected cells may be spotted directly onto "subbed" slides. These can be fixed (as described in step two), dried, and stored indefinitely.

A good concentration for cell suspensions to be smeared is approximately 1×10^7 cells/ml. Again, one can use cells previously fixed in 3.7% formaldehyde/1X PBS. Spot 10 μ l of the well-mixed cell suspension directly onto the slide. Use the backside of a 200 μ l pipette tip to spread the cells. Let the smear air dry.

2) Post-Fixation

Place slides in a slide rack. Set in a staining jar containing a mixture of methanol:formaldehyde, 9:1. (Final concentration of formaldehyde is 3.7%). Incubate at room temperature 20 minutes. Remove slides and dip briefly in H₂O to rinse. Air dry.

3) Hybridization Buffer (make fresh, as follows:)

<u>Stock reagent</u>	<u>Volume to add</u>	<u>Final Conc.</u>
dH ₂ O	2.76 ml	-----
25X SET	800 μ l	5X SET
2% BSA (optional)	400 μ l	0.2% BSA
10% SDS	40 μ l	0.1% SDS

4) Hybridization

Lyophilize enough fluor-labeled oligodeoxynucleotide probe for 50 ng probe/smear. Resuspend the probe in Hybridization Buffer (above) to a final concentration of 5.0 ng probe/ μ l in Hybridization Mix (from above). 10 μ l of this probe dilution is used per smear, for a total of 50 ng probe/smear. Mix probe and hybridization mix gently by tapping the tube lightly -- try to avoid vigorous agitation and bubble formation.

Add 10 μ l of the appropriate probe onto each of the smears, directly on top of the cell smear. Next the slides are carefully placed in an airtight chamber containing a tissue soaked in Hybridization Buffer, to prevent drying of the probe-hybridization mixture. For single slides, 50 ml Falcon tubes make convenient hybridization chambers. Place the slide in a 45°C dry type incubator, and hybridize from 2 hours to overnight. (The hybridization and wash temperatures are determined empirically for each different probe.)

5) Washes (Can be done after 2-16 hours of hybridization.)

Set up a staining jar in the 45°C waterbath (the wash temperature will depend on the particular probe being used) and fill it with pre-equilibrated 0.2X SET. Place the hybridization slides in the 0.2X SET. Note the time; after 10 minutes, pour off the 0.2X SET and replace with fresh 0.2X SET. Wash for 10 more minutes, and again replace with fresh 0.2X SET. After a final 10 minutes, remove slides and allow to air dry in subdued light. When slides are dry, place a small drop of mountant directly on the smear, cover with a coverslip; view via epifluorescence microscopy.

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**Application of Molecular Genetic Techniques
to Microbial Ecology**
[overheads from lecture]

**BIODIVERSITY & COMMUNITY STRUCTURE:
WHO'S OUT THERE???**

**POPULATION STABILITY & DYNAMICS:
HOW DO POPULATIONS CHANGE,
& WHAT FACTORS CONTROL VARIABILITY?**

**ORGANISMS to PROCESSES:
WHO'S DOING WHAT (AND HOW)?**

ORGANISMAL ADAPTATIONS/INTERACTIONS

BIODIVERSITY/COMMUNITY STRUCTURE: WHO'S OUT THERE?

SPECIFIC QUESTIONS:

What is the species composition of specific microbial populations?

Do we ignore a significant fraction of extant microbial diversity?

On what spatial scale are communities appropriately defined?

POTENTIALLY USEFUL MOLECULAR APPROACHES:

**Cloning and sequencing of phylogenetically informative genes
from mixed, microbial assemblages**

Identification of species or groups with nucleic acid probes

Identification of species or groups with immunological probes

POPULATION STABILITY & DYNAMICS: HOW DO POPULATIONS CHANGE, & WHAT FACTORS CONTROL VARIABILITY?

SPECIFIC QUESTIONS:

How do individual species vary in time and space?

What are the major factors controlling this variability?

Physical

Temperature

Light

Pressure

Chemical

Nutrients

Anthropogenics

Redox

Biological

Predation

Viral infection

Syntrophy/symbiosis

What is the extent and role of intraspecific variability?

POTENTIALLY USEFUL MOLECULAR APPROACHES:

Identification and enumeration using nucleic acid probes

Identification and enumeration using immunological probes

Molecular analyses of acclimation and stress responses

RFLP analysis of intraspecific genetic variability

Nucleic acid sequence analyses of genetic variability

ORGANISMS to PROCESSES: WHO'S DOING WHAT?

SPECIFIC QUESTIONS:

Can functional roles be inferred from phylogenetic affiliations?

Can organism variability be correlated with process variability?

Can we monitor the expression and regulation of functional genes in the environment?

**Regarding regulation of specific processes in the environment,
what are the relative roles of:**

- 1) Major changes in community structure**
- 2) Intraspecies variability and selection of genetic variants**
- 3) Intracellular regulatory circuits**

POTENTIALLY USEFUL MOLECULAR APPROACHES:

**Monitoring species variability with probes (& correlations with
environmental activities)**

**Measure environmental gene expression with mRNA specific or
immunological probes**

**Follow induction of reporter genes in genetically engineered
organisms**

Molecular analyses of intracellular regulatory circuits

ORGANISMAL ADAPTATIONS/INTERACTIONS

SPECIFIC QUESTIONS:

What specific adaptations are required, at the genetic and molecular level, for optimal function in unique marine habitats?

What is the role of gene regulation in acclimation/adaptive responses?

What molecular mechanisms are responsible for recognition, communication, and maintenance of host/symbiont relationships?

POTENTIALLY USEFUL MOLECULAR APPROACHES:

Structure/function comparison of nucleic acid and protein sequences

Gene fusion experiments for monitoring gene regulation

Genetic analysis of mutants/variants in host/symbiont studies

Use of molecular probes to follow symbiont transmission to host progeny

An Overview of the Importance of Subtidal Sediments to Nitrogen Cycling in Coastal Ecosystems

A variety of evidence has shown that the primary production of most nearshore coastal ecosystems is limited by nitrogen. For example, there is a strong relationship between nitrogen loading and primary production in estuarine ecosystems (Boynton *et al.*, 1982; Nixon and Pilson, 1983). In contrast, phosphorous appears to be the most limiting element for primary production in freshwaters. A variety of reasons for the differences between freshwater and marine systems have been proposed. Howarth *et al.* (1988) have suggested that the greater importance of planktonic nitrogen fixation in freshwater may explain why freshwaters are more likely to be P limited.

The sediments play an important role in recycling nitrogen in coastal ecosystems. Nitrogen is deposited onto the sediments as particulate detritus where it is mineralized to ammonium and diffuses into the water column, then becoming available for uptake by plankton. The amount of recycling varies considerably among systems (Boynton *et al.*, 1982; Kelly, in press), but in most estuaries and coastal systems nitrogen is used several times before it is lost by flushing, burial, or conversion of N₂.

Burial of nitrogen in sediments is fairly low; usually less than 10% of the annual nitrogen input to the sediments is buried. In contrast, losses by denitrification have been reported to be high. Seitzinger (1988) has estimated that 20-70% of the nitrogen input to the sediments may be lost via denitrification.

To predict the magnitude and seasonal cycle of primary productivity it is necessary to understand the controls on nitrogen release from sediments. A seasonal study at one site in Buzzards Bay showed that most of the variation in nitrogen release from the sediments could be explained by temperature and phaeopigment concentration (Banta, 1991). In contrast, much of the difference between sites can be explained by differences in input to the sediments and the amount of denitrification (Banta, 1991; Kelly, in press).

There are two mechanisms by which denitrification takes place in sediments. In some sediments nitrate is taken up by the overlying water and denitrified. The controls on this mechanism of denitrification are the nitrate concentration of the overlying water and organic matter availability of the sediments. The more important mode of denitrification is coupled nitrification/denitrification. In this case, mineralized ammonium is nitrified in the oxic portions of the sediments. A portion of this nitrate diffuses into the anoxic areas of the sediments where it is subsequently denitrified. (See Vanderborght *et al.*, 1977 for a model of this process.) Controls on this process are complex and include the depth of the oxic layer, the irrigation

rate of the sediment, and the availability of organic matter below the oxic layer.

A gradient from the sewage outfall in Boston Harbor to Massachusetts Bay illustrates how the importance of these two modes of denitrification change with organic matter loading (Giblin *et al.*, 1990). In Boston Harbor, sediments are anoxic to the surface and take up nitrate from the overlying water. In Massachusetts Bay the sediments have a well developed and bioturbated oxic layer. These sediments release both nitrate and ammonium. The ratio of oxygen consumed to nitrogen released suggests that a substantial amount of the N mineralized in these sediments is lost via denitrification.

The Importance of Benthic Macrofauna to Decomposition and Nitrogen Cycling in Sediments

Benthic macrofauna have the potential to change the decomposition and cycling rates in sediments through their activities (Aller, 1982). By building irrigation burrows animals change the availability of oxygen in the sediments and remove potentially inhibitory end-products. Macrofauna redistribute particles, break them up, and repackage them into fecal pellets. It has been proposed that by grazing senescent bacteria from particles, macrofauna increase decomposition rates by keeping microbes in log-phase growth. Animal activities also change sediment surface characteristics and alter resuspension. In laboratory experiments, adding macrofauna to decomposing detritus usually increases decomposition rates (Aller, 1982).

Recent experiments by Banta (1991) have indicated that the role of macrofauna in altering decomposition rates may be more complicated than previously believed. When macrofaunal populations were manipulated in sediment taken from Buzzards Bay in the summer, the presence of benthic animals increased decomposition by magnitudes of 2 to 3; however, when sediments were taken from the field in the fall, the macrofauna had no effect on decomposition. Macrofauna did change porewater irrigation in both experiments. To test whether the substrate quality in sediments explained the difference between the two experiments, Banta (1991) manipulated both organic matter and macrofauna numbers. When organic matter quality was very low or very high macrofauna did not alter decomposition rates. Macrofaunal stimulation of decomposition did occur when organic matter was of intermediate quality.

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Heterocyst Differentiation and Nitrogen Fixation in Cyanobacteria

Cyanobacteria are ubiquitous prokaryotes that cover Earth, inhabiting the oceans, streams, ponds, soil and rock surfaces. They carry out green plant photosynthesis, liberating oxygen in the light. Many species also convert atmospheric nitrogen to ammonia, enabling them to live and reproduce in environments that provide little more than phosphate, sulfate, and a few metal ions. Most nitrogen fixing cyanobacteria grow in long filaments of more than 100 cells. When nitrate or ammonia are abundant, all the cells look the same. When these sources of nitrogen are limiting, cells specialized for nitrogen fixation differentiate at regular intervals along the filament. In our strain of Anabaena, approximately every tenth cell differentiates.

Anabaena vegetative cells contain 10-20 copies of a circular chromosome of 6.4 Mb. We estimate, on the basis of RNA hybridization studies, that 20-25% of the chromosome, corresponding to 1200-1600 genes, is required for the processes of heterocyst differentiation and nitrogen fixation.

To characterize these genes we have taken three approaches: cloning and analysis of genes known to be differentially regulated; isolation of developmental mutants and the genes that complement those mutations; and the brute force description of clones in stage-specific cDNA libraries prepared from mRNA populations. Each approach has yielded some unexpected results; one example of each will be described.

Nitrogen fixation is accomplished by the enzyme complex called nitrogenase, which contains three different polypeptides, several iron-sulfur clusters, and cofactors of iron, sulfur, and molybdenum. The nitrogenase polypeptides are encoded by three genes called nifH, nifD, and nifK which, in most diazotrophs, are co-transcribed in the order nifHDK. This operon is not transcribed in cells growing on ammonia or in the presence of oxygen, due to a requirement of the nifH promoter for activation by a DNA-binding protein (NifA) that is not present or not functional except under nitrogen starvation, anaerobic conditions. One of the reasons for heterocyst differentiation is to provide a locally anaerobic environment for both nif gene expression and nitrogenase function. When we originally cloned and sequenced the nifH, D, and K genes from the DNA of Anabaena vegetative cells, we were surprised to find an 11-kb element interrupting the coding sequence of the nifD gene. The presence of this element would make it impossible to transcribe the nifK gene and therefore impossible to fix nitrogen. Subsequently, it was discovered that the 11-kb element is excised from the chromosome during heterocyst differentiation by site-specific recombination between directly repeated sequences at the ends of the

element. The excision reaction results in the formation of a non-replicating 11-kb circular DNA molecule and a fused chromosome, in which the nifHDK operon is restored with an intact nifD reading frame. The restored operon is the template for synthesis of nifHDK messenger RNA.

The excision reaction is catalyzed by a recombinase enzyme encoded within the 11-kb element itself. Mutation of the xisA gene prevents excision and results in a Nif-phenotype. The 11-kb element is widely distributed in free-living Anabaena, having been identified in newly isolated strains from India, Costa Rica, and the U.S. Since the element must be excised for the strain to survive nitrogen starvation, there must be another function it encodes that provides a selective advantage to cells carrying it. That function is still unknown, although it has been guessed to be a phage immunity substance, a restriction activity, or a DNA repair activity.

Since nitrogen fixation requires the anaerobic milieu and other properties of the heterocyst, Anabaena mutants affected in heterocyst development are unable to grow on N₂ as nitrogen source in air. It has therefore been possible to design a penicillin-selection scheme, based on the Nif-phenotype, to select mutants defective in heterocyst differentiation. There are many morphological classes of mutants, including one that makes too many heterocysts, one that makes too few (only at the ends of filaments) and one that makes none. To illustrate this approach, the latter will be described further.

Mutant 216 grows perfectly well on nitrate or ammonia as nitrogen source. Starved of nitrogen, it turns yellow and dies. It does not initiate heterocyst differentiation. A single gene called hetR, transferred from a library of wildtype DNA fragments by conjugation, complements the mutation in mutant 216. The hetR open reading frame has no similar counterpart in any current database of protein sequences and has no known protein motifs. The gene is transcribed early during heterocyst differentiation and is not required for vegetative growth. When extra copies of the wildtype hetR gene are introduced into wildtype cells, too many heterocysts differentiate and the culture dies. We believe that the gene product participates in a regulatory cascade that triggers differentiation, perhaps by binding a diffusible inhibitor of differentiation. We hope that other elements of the cascade can be identified by the characterization of pseudorevertants of the lethal extra-copy phenotype just described.

The last approach starts with the isolation of libraries of cDNA fragments representing the genes expressed uniquely during particular stages of heterocyst development (Christopher Bauer, personal communication). This has been accomplished by the preparation of total RNA from cells, say, early after the initiation of differentiation as well as from cells growing on nitrate or ammonia medium. Single strand cDNA is made from the first RNA by reverse transcription, using oligonucleotide primers. The single stranded cDNA is then saturated with an excess of RNA

prepared from the control cells. Finally, the few uncovered cDNAs are made double-stranded by oligo priming again, this time using DNA polymerase. The final double-stranded cDNAs are cloned into a plasmid vector. The resulting mini-libraries contain copies of the genes transcribed at specific stages of heterocyst development. So far, more than 200 unique clones have been characterized.

The cDNA to be described in detail was found to be similar in sequence to the nifJ gene of Klebsiella, which encodes a 120-kD iron-sulfur protein that transfers electrons from pyruvate to flavodoxin en route to nitrogenase. The cDNA was used to clone the entire nifJ gene from a cosmid library of Anabaena DNA. The Anabaena nifJ sequence is very similar over its entire length (3.3 kb) to Klebsiella nifJ, except that it contains an insert of five tandem repeats of a heptamer, resulting in an extra loop of 12 amino acids in the Anabaena nifJ gene product. Multiple copies of the heptamer repeat occur in the Anabaena chromosome, but all of the other known occurrences are at the ends of genes. At present there is no sure explanation for these short tandemly repeated elements throughout the Anabaena chromosome. One possibility is that they are the fossil footprints of a transposable element that once traveled through the chromosome.

In summary, the study of cellular differentiation in a simple two-component prokaryotic system has produced three unanticipated results: developmentally regulated rearrangement of genes involving excision of interrupting elements; short tandemly repeated elements inserted into the body of at least one gene; and a complex regulatory cascade linking cellular differentiation to environmental cues.

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Enzymes of Nitrification and Denitrification: Parts of the Global Nitrogen Cycle

Within the nitrogen cycle, autotrophic nitrification is carried out aerobically by only about 5 genera of bacteria, each having only a few (or one) species. The ammonia oxidizers, exemplified by Nitrosomonas, produce nitrite (NO_2^-) as a final product. The oxidation of nitrite to nitrate (NO_3^-) is promoted by one genus, Nitrobacter. Denitrification, the stepwise reduction of nitrate to N_2 , is carried out anaerobically by a large number of genera (twenty-some) of facultative aerobic and photosynthetic bacteria. Ammonia oxidizers can also function as denitrifiers under conditions of O_2 deficiency.

The nitrifiers convert NH_3 to NO_3^- with use of three critical enzymes: ammonia monooxygenase and hydroxylamine oxidase (dehydrogenase, actually) within the ammonia oxidizers, and nitrite dehydrogenase within Nitrobacter. Denitrification depends on four induced enzymes: nitrate reductase, nitrite reductase, nitric oxide (NO) reductase, and nitrous oxide (N_2O) reductase.

Ammonia monooxygenase -- This enzyme is an, as yet, poorly characterized membrane-bound enzyme that uses O_2 in a reaction that inserts one O-atom into NH_3 to make hydroxylamine (NH_2OH) and reduces the other O-atom to water. The reducing agent for the reductive reaction is in fact NH_2OH , the product of the O-insertion reaction. N_2H_4 can substitute for NH_2OH as reductant. The reaction can be written as: $\frac{1}{2} \text{NH}_2\text{OH} + \text{NH}_3 + {}^{18}\text{O}_2 + \frac{1}{2} \text{H}_2\text{O} \text{ ----} > \text{H}_2{}^{18}\text{O} + \text{NH}_2{}^{18}\text{OH} + \frac{1}{2} \text{NO}_2^- + \frac{1}{2} \text{H}^+$. Ammonia monooxygenase is not specific for NH_3 and in fact can insert O into a variety of other compounds, including CH_3 , CO, benzene, and cyclohexane. It is inhibited by nitrapyrin (2-chloro-5-trichloromethylpyridine) and by Cu chelators. This latter property, and bleaching at 350-400 nm under UV light, provides indirect evidence that the metal center is a Cu_2 pair, as is found in tyrosinase. But the existence of an Fe_2 center, as found in methane monooxygenase, cannot be entirely ruled out. The electrophilic hydroxylating species would be $\text{Cu(II)}=\text{O}$, a cupric oxene donor. This species can be thought of as a Cu-stabilized O-atom and is analogous to the ferryl oxene, $\text{Fe(III)}=\text{O}$, intermediate in P-450 hydroxylations.

Hydroxylamine dehydrogenase -- The reaction of this periplasmic enzyme can be written as: $\text{NH}_2\text{OH} + \text{H}_2\text{O} \text{ ----} > \text{NO}_2^- + 4\text{e}^- + 5\text{H}^+$, in which the second atom of O in nitrite is derived from water, not O_2 . Nitrogen undergoes a 4-electron oxidation, and one of the clever features of the enzyme is that oxidation occurs without release of intermediates. The enzyme has an α_3 structure. Each subunit contains 7 covalently bound

heme c groups of widely different redox potentials and about three residues of a heme-like Fe-macrocycle (P-460). The latter centers would appear to bind NH_2OH and carry out the primary oxidation, with the hemes c receiving electrons from P-460 and donating them through cytochrome c-554 to ammonia monooxygenase and cytochrome c oxidase (cytochrome aa₃). Although the mechanism of oxidation of NH_2OH is unknown, the ease of oxidation of NH_2O^- and its carbanion-like character make it likely that the Fe of P-460 coordinates NH_2OH , which is then activated by proton abstraction.

Nitrite dehydrogenase -- This membrane-bound enzyme of Nitrobacter oxidizes nitrite to nitrate. This is a tough way to make a living! $\text{NO}_2^- + \text{H}_2\text{O} \rightarrow \text{NO}_3^- + 2\text{e}^- + 2\text{H}^+$. The enzyme contains Mo and has been shown by ¹⁸O, ¹⁵N-isotope experiments to catalyze the reversible interconversion between nitrite and nitrate by way of an O-atom (2-electron) transfer mechanism. The relevant redox states of Mo in this reaction are probably (IV) and (VI), with Mo(VI)=O as the oxene carrier. The Mo-oxene allows transfer of an O-atom from nitrate to nitrite as well as the protons.

Nitrate reductase -- This dissimilatory enzyme is membrane-bound, contains Mo and Fe_4S_4 centers and has an $\alpha\beta$ structure. Sometimes a third peptide (cytochrome b-containing) is found to be associated with the enzyme. The active site is the Mo center, and the Fe_4S_4 and cytochrome b serve a role in electron transfer to and from the Mo center. The Mo atom is coordinated by a pterin. Redox cycling involves Mo(IV) and (VI). In addition, Mo(V) can often be detected by EPR. The redox mechanism is unknown, but may well involve O-atom transfer by analogy with nitrite dehydrogenase. In addition to the reduction of nitrate to nitrite, the enzyme can also reduce chlorate and bromate to Cl^- and Br^- , respectively, and nitrite to NO. The latter is a slow reaction involving a 1-electron (not 2) reduction.

Nitrite reductase -- Denitrifiers produce two different kinds of NO-producing dissimilatory nitrite reductases. One is a cytochrome cd₁. Each subunit of this dimeric enzyme contains one covalently bound heme c and one noncovalently associated Fe-dioxoacryloisobacteriochlorin (heme d₁). This enzyme can also function as an oxidase. The site of binding O_2 and nitrite is the heme d₁. The other type is a Cu-containing enzyme. The Cu-enzyme from Achromobacter cycloclastes has been crystalized and a high resolution structure calculated. The enzyme has an α_3 structure with 2 Cu-atoms per subunit. One Cu-atom is coordinated to 2-histidine N, 1-cysteine S, and 1 (distant) methionine S. The second Cu-atom, where nitrite binds, is coordinated to 3-histidine N and 1-solvent O. There is considerable evidence that the redox cycle of both of these types of nitrite reductase involves an electrophilic species of NO (e.g., HNO_2 or H_2NO_2^+). The enzyme can catalyze the nitrosation (nitrosyl, NO^+ , transfer) of nucleophiles, such as NH_2OH and N_3 , and the exchange of O between nitrite and water. Increasing the electrophilicity of the NO group of nitrite by protonation or metal coordination at the second O is a rational strategy for

activation of nitrite for reduction, inasmuch as it simultaneously decreases the basicity of the leaving O-atom and lowers the electrostatic barrier for injection of an electron or hydride. Both kinds of nitrite reductase are found in the periplasmic space of Gram negative denitrifying bacteria.

Nitric oxide reductase -- This membrane-bound enzyme is a cytochrome *b_L*, *c* complex composed of two different peptides. It may contain some non-heme iron as well. The complex reduces NO by 1 electron to N₂O. Although the mechanism is not established, one simple and attractive mechanism involves reduction of NO to NO⁻ (nitroxyl or nitrosyl hydride), which is known to spontaneously and rapidly protonate, dimerize, and dehydrate to form N₂O.

Nitrous oxide reductase -- This periplasmic enzyme is generally a dimer and contains 4 Cu-atoms per subunit. Two of these exist as a Cu(I)-Cu(II) pair in the oxidized enzyme. The involvement of Cu in this enzyme is curious due to the extreme unreactivity of N₂O. Although N₂O is reduced by Co(I) and Zn(I), there is no precedence for reduction of N₂O by Cu(I). In addition, the only stable metal-N₂O complex is [Ru(NH₃)₅ N₂O]²⁺. The mechanism of the enzyme is not only unknown but may well involve precedence-breaking chemistry. It was suggested in the lecture that the Cu(I)-Cu(II) pair in the enzyme may function as a poor man's ruthenium. The Cu(I) is hypothesized to π -complex N₂O at the N₂ end, and this interaction is reinforced by coordination of Cu(II) with the O-atom at the negative dipole end of N₂O. This latter coordination would also serve to stabilize the O-radical anion generated following 1-electron reduction of N₂O and N-O bond breaking.

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Use of Bulk Stable Isotopes for Environmental Studies: Stable Isotopes at the Molecular Level

Stable isotope compositions of organic substances are exceedingly powerful tools in the assessment of the origin, fate, and history of a material. By determining the isotopic compositions of organisms and their potential diets, quantitative assessment of foodweb relationships and trophic positions can be made. This assessment is achievable primarily through the understanding that an enrichment of approximately 3 parts per thousand occurs in nitrogen isotopes with each trophic level. Differences are also resolvable between organisms that derive nutrition from either C3 or C4 types of plants as a result of highly discernable associated fractionations of the carbon isotopes by those plants in the primary incorporation of carbon. Bulk measurements of natural or anthropogenic pollutants can also be assessed as to source through carbon isotope signatures (in oil spills, for example) or nitrogen isotope compositions (in nitrate contamination of ground waters by fertilizers).

Bulk materials are really mixtures of hundreds to thousands of chemical components, each having its own isotopic composition. The relative contribution of each of these signatures to that of the bulk material is quantifiable through mass balance or isotopic proportionation equations. Over the years, numerous attempts have been made to isolate individual molecular components using liquid or gas chromatographic techniques in order to better interpret or trace an organic material's history or source. The possibility of comparative biochemistry in modern or fossil organisms has been suggested through assessing isotopic differences between compounds of a family of components. Such differences are the result of enzymatic fractionation effects during synthesis or metabolism of the compound; an example of such an effect has been clearly seen using the enzyme transaminase, with nitrogen isotope fractionations observed in acetyl-glucosamine and in the amino acids ASP and GLU (and others) in both cultured and natural populations of organisms. Isotopic compositions of individual hydrocarbons have the potential for establishing bacterial sources for the materials, and have been useful in correlation techniques both in the petroleum industry and in pollution assessment. Individual carbohydrate isotope compositions also show great potential in metabolic and diagenetic studies. Depletions in the carbon isotopes of reaction products allow for calculations that quantify use and production of new organic materials and that resolve them from native materials, even though the chemical compositions of the substances are identical. Through recent technological advancements, gas chromatographic (GC) effluents can be pyrolyzed and the resulting carbon dioxide directly introduced into the stable

isotope ratio mass spectrometer (IRMS). This modification, GC/IRMS, allows for rapid analysis of the carbon isotopes on components in a mixture, and with increased sensitivity, on the order of 0.5 nM, of each compound.

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**Energy Metabolism During Larval Development and
Animal/Chemical Interactions in the Sea**

Animals that live in seawater are in a medium that contains organic carbon and nitrogen in dilute solution. Larval forms of soft-bodied marine invertebrates are adapted to take advantage of the fact that most of the organic nutrients in their environment are in solution as dissolved organic material (DOM). Evidence for the importance of DOM to metazoans was presented by showing that larval forms can increase in biomass, even in the absence of particulate foods. The physiological basis for using DOM as an energy source is dependent upon an increased transport capacity for DOM as growth proceeds. Mass coefficients and exponents were determined for 1) alanine transport rates, and 2) metabolic rates, and these coefficients were not statistically different when determined over the life span of a larva. Thus, as growth proceeds, larvae increase their ability to obtain a potential supply of metabolic fuel (DOM) in direct proportion to the increase in their metabolic demand. The percent of this increased transport capacity that larvae could actually utilize in nature will depend upon the substrate concentrations in their environment. Current views on what these concentrations are in seawater may be altered as more attention is given to the fine scale distributions of organic chemicals in the ocean.

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Stable Isotopes and Their Use in Biogeochemical Studies

The natural abundance of ^{15}N ($\delta^{15}\text{N}$) can provide insights into the nitrogen cycle in planktonic ecosystems on temporal and spatial scales that are difficult to study using traditional experimental approaches. On average, the natural abundance of ^{15}N is approximately 0.368% by atoms, though significant deviations from this mean value characterize many of the biologically active pools of nitrogen in marine systems. These differences in $\delta^{15}\text{N}$ are the result of the isotopic discrimination associated with many biologically mediated transformations of nitrogen. An understanding of the degree and pattern of isotopic fractionation associated with specific reactions is essential to the use of $\delta^{15}\text{N}$ data in studying natural systems, and can provide insights into the mechanisms that underlie that fractionation. For example, measurements of isotopic fractionation during NO_3^- uptake by six phytoplankton species grown in continuous culture revealed no significant intraspecific variation, though diatoms consistently exhibited higher fractionation factors than flagellates. These results suggest that phytoplankton communities dominated by diatoms may have significantly different isotopic signatures than communities dominated by flagellates. In addition, these results suggest that isotopic fractionation occurs primarily during transport of NO_3^- across the plasmalemma.

Samples collected during a field study of the nitrogen cycle in the Chesapeake Bay revealed no consistent bay-wide pattern in the $\delta^{15}\text{N}$ of planktonic and dissolved nitrogen during the spring and fall of 1984, though the $\delta^{15}\text{N}$ of plankton generally increased with trophic level. In the spring, the $\delta^{15}\text{N}$ of NO_3^- in the surface layer of the bay showed significant spatial variations related to the nonconservative behavior of NO_3^- as a result of biological activity. The relationship between the $\delta^{15}\text{N}$ and the net deficit of NO_3^- within the surface layer implied that the consumption of NO_3^- was accompanied by a fractionation factor of ca. 7%. This fractionation factor is within the range of values measured for NO_3^- uptake by phytoplankton, but is much lower than the fractionation that accompanies denitrification. Significant changes in the $\delta^{15}\text{N}$ of the dissolved and planktonic pools occurred in the fall after an intense storm promoted vertical mixing and the injection of NH_4^+ into the surface layer of the bay. The magnitude of these changes reflected both isotopic fractionation and the biological turnover time of nitrogen within the plankton.

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Molecular Mechanisms Controlling Settlement and Metamorphosis of Marine Invertebrate Larvae

Planktonic larvae of many benthic species require a specific chemical "signal" from the environment to induce settlement and metamorphosis. In larvae from representatives of three phyla, the molecular signals and mechanisms controlling substratum-specific recruitment from the plankton are similar to (or cross-react with) those controlling behavior and development in other animals, including humans. The inducer recognized by Halotis rufescens (red abalone; mollusc) larvae is a GABA-mimetic peptide produced by the crustose red algae on which the larvae settle; this peptide also binds strongly and specifically to GABA receptors from mammalian brain. Enzymatic "fingerprinting" shows that the inducer recognized by Agaricia humilis (coral) larvae is a sulfated glycosaminoglycan (polysaccharide) found in the cell walls of the crustose red algae on which these larvae settle; this molecule is mitogenic for mammalian lymphocytes. The inducer recognized by the larvae of Phragmatopoma californica, a reef-building polychaete, appears related to DOPA and thyroxine. This inducer is found in the DOPA-protein adhesive used by the adult worms to form their tubes; contact-dependent recognition of this substance triggers "gregarious" recruitment of the larvae, leading to the formation of massive aggregations and reefs. Experiments in the field show that larval recognition of these inductive signal molecules is responsible, in part, for the spatial patterns of recruitment of these three species in the natural environment.

Site-specific settlement, metamorphosis, and the activation of differential gene expression in abalone larvae are controlled by two convergent chemosensory pathways, responsive to two different classes of chemical signals from the environment, by a mechanism similar to long-term potentiation in mammalian brain. Both the inductive algal peptide and GABA bind to externally accessible chemosensory receptors controlling the "morphogenetic pathway." Activation of these receptors is apparently then transduced by intracellular changes in cAMP, Ca^{++} , and PKA activity, leading to an efflux of Cl^- across the chemosensory membrane. The resulting excitatory depolarization is thought to transduce the morphogenetic chemical signal from the environment to an electrochemical signal propagated by the larval nervous system. Sensitivity of this morphogenetic pathway can be amplified 100-fold by low concentrations of lysine dissolved in seawater, perhaps enhancing settlement in favorable areas.

The "amplifier pathway" includes transmembrane lysine receptors reciprocally coupled to a G protein-diacylglycerol-PKC cascade. These components retain their function *in vitro*, in chemosensory cilia partially

purified from the larval epithelium. These cilia also contain mRNA; conversion to cDNA, amplification by PCR, cloning, and sequence analysis reveal that this mRNA codes for a signal transducing G protein with sequence homology to the G_q recently found in mammalian brain. Linkage of these pathways to the activation of specific gene transcription during metamorphosis is under investigation. These studies required the development of improvements in the procedures for purification and characterization of nucleic acids from marine species. Practical applications have helped make abalone aquaculture profitable in California, and are improving cultivation of other valuable species. Potential applications to medicine are under investigation.

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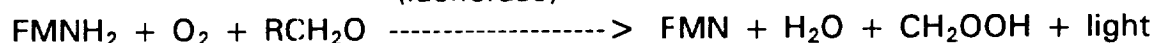
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Ecology of Luminous Bacteria

The luminous bacteria are a widely distributed group of Gram-negative eubacteria united by one element -- the ability to emit visible light. As far as is known, all of these bacteria utilize the same mechanism for light emission, which is a mixed function oxidase type reaction catalyzed by the enzyme bacterial luciferase. Bacterial luciferase is an alpha beta dimer of approximately 80,000 molecular weight, which catalyzes the reaction shown below, in which flavin mononucleotide and a long chain fatty aldehyde are both oxidized by molecular oxygen, giving sufficient energy for emission of blue-green light (Baldwin & Zeigler, 1992). This enzyme is uniquely bacterial, except in those cases in which the luminous bacteria are symbiotically associated with eukaryotes (Nealson & Hastings, 1991).

(luciferase)



The genes that code for the alpha and beta subunits of luciferase have been termed *luxA* and *luxB*, and have been cloned and sequenced from several different strains of luminous bacteria. Sequence analysis has shown that the two subunits are of similar origin, and that all bacterial luciferases so far analyzed are structurally related to each other. Three other structural genes, *luxC-E*, code for proteins involved with the supply of long chain aldehyde. These genes are also found in all strains of luminous bacteria examined genetically. The five structural genes also share a similar gene arrangement, CDABE, although in various strains and species some other open reading frames of unknown function are known (Meighen, 1988 & 1991).

Several species of luminous bacteria are found in the genera *Vibrio*, *Photobacterium*, *Shewanella*, and *Xenorhabdus* (Akhurst & Boemare, 1990). All are closely related species, clustered into a small region of the gamma subgroup of the proteobacteria. The absence of luminescence (and apparently of luciferase) in other eubacteria suggests that this process is a rather recent evolutionary event. With the exception of *Xenorhabdus*, which is a group of bacteria symbiotic with nematodes, almost all luminous bacteria are marine in their distribution, a fact that is not understood ecologically, but which is thought by many to be related to the widespread use of bioluminescence by marine eukaryotic organisms, with which the luminous bacteria are often associated symbiotically.

While luminescent bacteria can be found planktonically throughout the world's oceans as freeliving or planktonic forms, the numbers are usually quite low, only a few per ml of seawater (Nealson & Hastings, 1991). Other niches include saprophytic, parasitic, gut symbiotic, and light

organ symbiotic, where cell populations can reach 10^9 ml⁻¹ or higher. The planktonic populations of most species of luminous bacteria are thought to be strongly impacted by their participation in these other niches (Nealson & Hastings, 1991). Many of the light organ symbionts are non-culturable -- apparently highly adapted symbionts that are not capable of living outside the host light organ (Haygood *et al.*, 1986, 1990, & 1992).

Molecular methods for the study of the ecology of the luminous bacteria have only recently been applied, but they have already had a strong impact. Ribosomal RNA sequencing has been done for almost all of the luminous species and has strongly supported the notion that this group is a recently evolved group of eubacteria in the gamma purple subgroup of the proteobacteria (Distel *et al.*, unpublished). The non-culturable light organ symbionts, while not identical species to the culturable forms, are closely related forms, which can be phylogenetically placed by rRNA sequence analysis (Haygood *et al.*, 1986, 1990, & 1992). While 16S rRNA probes have not yet been used to study the distribution and abundance of the luminous bacteria, enough sequence data are now available that these approaches should be applicable soon.

Using conserved portions of the *luxA* gene, Wimpee *et al.* (1991) showed that it was possible to amplify a 745 base-pair region from all species of luminous bacteria attempted. Furthermore, this 745 bp fragment, when labelled and used as a hybridization probe, was useful for identification of luminous bacteria. At low stringencies, such probes can be used to identify most *lux*-containing bacteria, while at high stringencies, they are species specific, hybridizing to only the species from which they were obtained. These have been used successfully for both laboratory and field identifications, and their application should greatly enhance the ability to study the distribution and abundance of the luminous bacteria, the first necessary step in understanding the ecology of this group.

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Molecular Methods for the Identification and Analysis of Phylogenetic Relationships Among Subsurface Microorganisms

We are developing group-specific 16S ribosomal RNA-targeted oligonucleotide hybridization probes for the rapid detection of specific types of subsurface microorganisms (e.g., groups of microbes that share certain physiological traits) (Balkwill *et al.*). Because portions of the 16S rRNA molecule are unique to particular organisms or groups, these unique sequences can serve as targets for hybridization probes with varied specificity (Olsen *et al.*). Target sequences for selected microbial groups can be identified by analysis of the available rRNA sequence data for microbes. Hybridization probes for these target sequences can be produced, and their effectiveness and specificity tested, with RNA dot blot and *in situ* hybridization (Giovannoni *et al.*). Selected probes are being used to study phylogenetic relationships among subsurface microbes and to classify these organisms into specific groups. Of particular interest is the relatedness of microbes residing in different geologic formations from an individual site and of microbes from different sites. The probes we are developing are also being used with *in situ* hybridizations to detect selected microbial groups in freshly collected subsurface sediments. An important advantage of *in situ* hybridization technology is that it permits detection of selected microbial types without the necessity of isolating and culturing them in the laboratory.

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Gene Expression in Symbiotically Associated and Free-Living Cyanobacteria

We are interested in obtaining a more complete understanding, at the molecular level, of interactions between the eukaryotic water fern Azolla and its prokaryotic endosymbionts -- the filamentous cyanobacterium Anabaena, and a variety of eubacteria. This association has gained considerable attention because of its ability to serve as a live nitrogen fertilizer in agriculture. To study patterns of gene expression for the symbiotic cyanobacteria we have utilized immunoelectron microscopy and in situ hybridization techniques. More specifically, we have adapted an in situ hybridization procedure developed for plant tissue to localize mRNA transcripts of key enzymes and proteins involved with nitrogen and carbon fixation (encoded by nif, glnA, rbcLS, and psbA genes) within cyanobacterial cells isolated from the Azolla-Anabaena association. The use of this in situ hybridization procedure has recently been extended for transcript localization in free-living filamentous cyanobacteria and should prove valuable for other prokaryotes.

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Remote Sensing of Ecosystems and Simulation of Ecosystem Processes

Very often we want to relate our understanding at one level of study (e.g., at the molecular level or microscopic level) to larger levels such as ecosystems or even global systems. To do this we have a number of options: 1) we can search for generalizations that permit us to identify key relationships and the variables that most describe them; 2) we can conduct sampling strategies that permit us to explain the natural variance and other statistical properties in the phenomenon being studied; 3) we can organize our understanding into simulation models using mathematical abstractions that are correlative or mechanistic; and 4) we can use other sources of related information, particularly spatial information, to help predict processes over larger regions of study. The latter option brings us to any form of remote sensing, the field of study defined simply to be the acquisition of information about an object without coming into direct contact with the object. This is as true for microscopic as it is for satellite observations, each of which can use the principles of the interaction of electromagnetic radiation with materials. In many studies of biogeochemical cycling in aquatic and in terrestrial ecosystems, we will use all of these options together, particularly as we attempt to build a predictive understanding of how ecosystems function and how to describe these functions over very large areas.

Remote Sensing

There are two main forms of remote sensing: so-called passive forms in which the source of radiation originates from an independent source such as the sun, and active forms such as radar in which the sensing system itself generates the radiation. In the laboratory, the source of radiation is almost always active, for example a monochromator in a spectrophotometric instrument. The source can also be the object itself, as emitted radiation in the thermal region of the spectrum. Remote sensing in the field and across large geographic regions involves either ground-based instruments, aircraft-borne sensing systems, or satellites. Satellite sensors often represent the culmination of years of development of sensors based upon extensive radiative transfer research beginning initially in the laboratory and passing through various levels of experimentation in the field and from prototype aircraft sensors. Biologists have a wide choice in remote sensing instruments from both aircraft and spacecraft. Table 1 shows the sensors planned for the Earth Observing System (EOS) and other sensors that can operate from aircraft.

Comparison of BIOME with planned EOS and existing aircraft sensors

	HIRIS	MERIS	MODIS-N	MODIS-T	
Bands	192	15	40	32	
Spectral range	400-2450	400-1050	413-14235	410-890	
Spectral resolution	~10	5-10	10-35 vis 20-50 ir	15	
Spectral sampling intervals	~10 contig.	discon.	discon.	~15 contig.	
GIFOV (m)	30	250-1000	428-856	1100	
Signal: Noise vis	300:1	1000:1	1200:1 red	800:1 blue	
Signal: Noise ir	120:1		110-310:1		
Global coverage (days)	~16	2-3	Daily	> 90% 2 days	
Tilt	+ -45 cross 52 fore 30 aft	nadir	nadir	+ -45 across track	
Swath	24	+ -40.8 1500	+ -55 of nadir (1500)	35.22 in track	
Dynamic range (bits)	n.a.	n.a.	n.a.	12	

	BIOME	AVIRIS	ASAS	GERIS	CASI
Bands	400	224	32	63	366
Spectral range	1100-2400	400-2450	465-871	500-2500	430-870
Spectral res.	~8	9-11	15	var.	~3
Spec. sampling intervals	~2 in 4 contig.	9-11 contig. 4 grat.	15 contig.	centered features	~3 contig.
GIFOV (m)	200	17	4.25	3.3 mrad @20K AGL	2-10
Signal: Noise vis	> 1000: 1	up to 100:1	100: flat	1500:1	200- 400:1
Global coverage (days)	30	100 km along path	n.a.	nadir	2 modes spatial spectral
Tilt	necessary	nadir	+ -45 @ 15 deg. fore/aft	nadir	nadir
Swath	50	10.5	0.45 (@5K AGL)	n.a.	1-5
Dynamic range	12-16	10	12-15	n.a.	12

Table 1: Comparison of proposed sensor (BIOME) with planned sensors for Earth Observing Systems (upper) and operating airborne sensors (lower) that have high spectral resolution capabilities. Not included are broadband sensors such as Landsat TM, AVHRR , and AOCI.

This list is by no means all-inclusive, but it shows the variety of higher spectral resolution capabilities. Some of these systems, such as the Compact Airborne Spectrographic Imager (CASI), which takes upwelling radiance measurements from water bodies in the visible range at a very high spectral resolution, are well suited for marine biological studies. CASI is a good choice for studying the largely unknown and highly variable upwelling radiance of freshwater bodies.

There are generally four attributes of ecosystems that are subject to study by remote sensing: spectral, spatial, angular and radiometric. Utilizing these four attributes, research has been done or continues to be underway to retrieve the ecosystem parameters shown in Table 2. These parameters have been put into five main groups: categorical or descriptive, biophysical, biochemical, environmental, and edaphic/topographic. For the remainder of this discussion, I will focus on two main parameters: biophysical and biochemical.

Biophysical:

The interaction of radiation from the sun with plant materials involves three processes: specular reflection from the waxy cuticle of the surface of leaves, volume scattering from inside the leaves, and absorption by various biochemicals. These three interactions are sketched in Figure 1(a). Once radiation passes through the cuticular layer, radiation encounters discontinuities in the index of refraction that are most acute at the cell wall-air interfaces of the cells, parenchyma and spongy mesophyll. The bending and reflection of radiation at these discontinuities produce diffuse scattering which may be further enhanced by smaller organelles within the cells. The radiation is attenuated as it passes through the leaves due to absorption. In the visible region of the spectrum, chlorophyll and its accessory pigments strongly absorb the photosynthetically active radiation (PAR). In the infrared region from 700 to 2500 nm, radiation absorption is weaker except near the absorption peaks of liquid water. These vibrational phenomena occur most strongly at 1900 nm, 1450 nm, and shorter wavelengths. Between 700 and 1100 nm, absorption is weakest in leaves and scattering of radiation dominates leaf optical properties. Radiation scattered back towards the source produces bulk leaf reflectance, while that scattered forward produces transmittance. Leaves are relatively transparent in this region so that as one stacks leaves on top of one another, the apparent reflectance of the stack continues to increase up to a depth of about 6-7 leaves. Electronic transitions in chlorophyll in the red region, on the other hand, attenuate most of the red radiation in just the parenchyma cells (Vogelmann, 1990). Thus, the ratio of the infrared reflectance to the red reflectance is proportional to the number of leaves stacked (see G. Asrar's book: Theory and Application of Optical Remote Sensing, John Wiley and Sons, New York).

REQUIREMENTS FOR VEGETATION, BIOLOGICAL, AND ECOSYSTEM SCIENCES

<u>Categorical</u>	<u>Biophysical</u>	<u>Biochemical</u>
Community type	Foliar biomass	Chlorophyll content
Understory conditions	Foliar water content	Accessory pigments
Understory phenology	Leaf area index	Nitrogen/protein content
Overstory phenology	Leaf shape/size	Lignin concentration
Architecture	Photosynthetic capacity	Carbohydrates
Successional composition	Absorbed PAR	Celluloses
Species types	Standing biomass, density (conducting/non-cond.; above/below ground)	
<u>Environmental</u>	<u>Edaphic/Topographic</u>	
Surface moisture in rooting zone	Soil texture	
Leaf and canopy temperature	Water holding capacity	
Surface air temperature	Elevation, slope and aspect	
Soil temperature		
Wind speed and direction	Drainage network and divides	
Aerodynamic roughness		
Solar insolation (direct vs. diffuse)	Parent material	
Surface absolute and relative humidity	Mineral/organic composition	
<u>In Situ Measurements</u>		
Trace gas fluxes		
Stream discharge and chemistry		

Table 2: Variables potentially sensible by the use of remotely sensed data that are useful in biological and ecosystem studies.

When considering leaves arranged into plant canopies as sensed by remote sensing instruments, other factors come into play. However, the physical basis of the ratio described above leads to methods to estimate the leaf area index of plant canopies. Leaf area index (LAI) is the total one-sided (projected) leaf surface area of all plants above a unit of ground surface. Two satellites provide consistent radiance measurements in the near infrared and red regions that have been related to the LAI of plant canopies. These are the Advanced Very High Resolution Radiometer (AVHRR) operated by NOAA and the Landsat system with two sensors, the Multispectral Scanner Subsystem (MSS) and the Thematic Mapper (TM). The ground spatial resolution of the AVHRR is one kilometer, but the resolution is often degraded to "four" kilometers by averaging the first four spatial elements, called picture elements or pixels, for each 3X5 group of pixels. These latter data were used by the Geosphere Project to produce the "first picture of Earth" commonly seen as a poster. The Landsat sensors have a spatial resolution of 60 and 30 meters, respectively, as well as additional bands and radiometric dynamic range. For purposes of estimating either the LAI or the absorbed PAR (APAR) of plant canopies from these data, researchers have concentrated on two indices, the simple ratio (SR) of the near infrared to red reflected radiance, or its transformation, called the normalized difference vegetation index (NDVI). These two are related by:

$$SR = NIR/RED \quad NDVI = \frac{NIR - RED}{NIR + RED} = \frac{(NIR/RED) - 1}{(NIR/RED) + 1} = \frac{SR - 1}{SR + 1}$$

The theory of Sellers (see Asrar's book) indicates that LAI should be proportional to the SR, but it becomes asymptotic with NDVI at LAI values of about 4-5. On the other hand, APAR is exponentially related to the SR but is nearly linear with NDVI. This is the reason for transforming a variable to obtain a more linear response to a desired quantity. For research using TM data in conifer forests, we found that LAI indeed increased nearly linearly with NIR radiance when the canopies were closed (>89% crown closure) and decreased exponentially with the RED as expected, while LAI was directly proportional to the SR (see Figure 2 for Oregon and western U.S. conifer forests; Peterson and Running in Asrar's book). However, as the canopies became more open, variations in background (vegetation of more reflective surface understory plants, bare soils/litter and rocks) degraded these relationships (Spanner *et al.*, 1990). When using the coarser spatial resolution data of the AVHRR, the influence of the background is reduced and similar relationships are produced. Further, the AVHRR passes over each point on the Earth two times daily while TM occurs only every 16 days. Thus, a time series of AVHRR data (being much less expensive than TM (\$100 versus \$4500 per scene) and covering much larger areas (1500 km versus 100X100 nautical miles) is possible. We and many others have

examined these time series, even globally (Tucker, 1986; Spanner *et al.*, 1990), to follow the phenology of plant communities in terms of a key biophysical parameter, APAR, as related to NDVI. You have probably seen many published examples of these time series on *NOVA* and in the scientific and popular press.

Atmospheric effects:

Between the sensor and the target is the Earth's atmosphere, a rapidly varying atmosphere that alters the radiance signal originating from the target. The atmosphere also alters the properties of the solar radiation reaching the target. The atmosphere scatters radiation as a function of wavelength, e.g., the scattered blue radiation responsible for the sky's color, and also absorbs radiation. The effects of the atmosphere on sensing from high altitudes are summarized in Figure 1(b). Radiation is scattered as it passes through the atmosphere, resulting in a direct beam and an indirect, diffuse irradiance upon surface targets. The radiation is also attenuated through absorption by various gaseous constituents, e.g., ozone, CO₂, and water. Water vapor absorption is so strong in the infrared region that virtually all of the solar radiation near 1450 nm, 1900 nm, and beyond 2500 nm is absorbed and never reaches the surface. The semi-transparent regions between these absorption bands are the "windows" through which optical remote sensing instruments make their measurements. Techniques are available to remove or reduce the contribution of the atmosphere on surface reflectance signals; most of them are dependent upon some independent measurement of atmospheric optical depth for accuracy. One must account for atmospheric effects when targets are located at different elevations, or when they are observed on different days or times of day or at various view angles, all of which will produce different path lengths or optical depths through the atmosphere. For more information on this, see Wrigley *et al.* (1990), Kaufman (1989, Asrar's book), and many other sources, including the atmospheric effects code developed by the Air Force Geophysical Laboratory (Kneisyz, 1983). The contribution of the atmosphere is more pronounced depending upon the reflectance of the target. For dark targets like water bodies and conifer forests, the atmospheric contribution to the upwelling radiance sensed can dominate the signal. For water studies, this contribution can make spatial variations in the atmosphere appear to be circulation patterns in the water. And, the absorption properties of the atmosphere can produce absorption features in the signal that can be confused with surface absorption features, e.g., atmospheric water vapor versus liquid water in plants (Gao and Goetz, 1990).

Fig. 1A

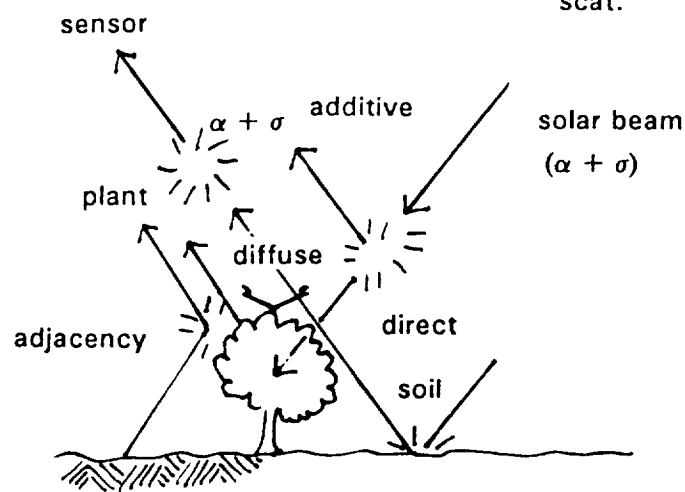
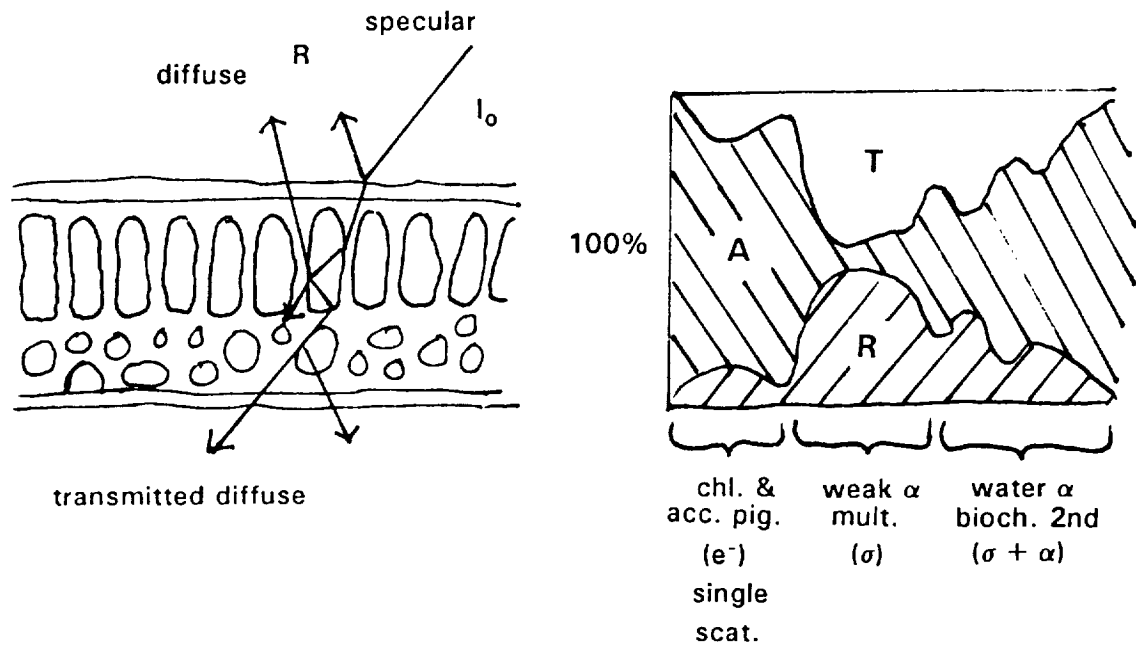


Fig. 1B atmospheric effects

Figure 1. (a) Schematic drawing of a leaf cross-section and the interactions of light-rays with cell walls, and, sketch of typical reflectance (R), transmittance (T) and absorption (A) of a leaf with the primary leaf characteristics which determine leaf optical properties. (b) The effects of scattering and absorption of solar radiation by the atmosphere and contributions to the overall signal received by the sensor.

Biochemical characteristics:

Until 1983, the conventional wisdom about what properties accounted for plant reflectance were those described above: cell wall-air interfaces caused scattering, chlorophyll and the accessory pigments absorbed visible radiation, and water content absorbed in the infrared. Independent research in the near infrared by the U.S. Dept. of Agriculture produced convincing evidence that the other biochemical compounds in leaves also can absorb radiation (Barton and Windham, 1988 and 1990; and many other references). The biochemicals consist of those involving nitrogen such as the proteins, the labile carbohydrates such as starch and sugars, the refractory carbon compounds such as lignin and cellulose, etc. Most of that work was based upon plant materials, such as forages, which had been dried (all the free water removed) and ground to a uniform powder. The principle involved here is that the organic bonds between the light atoms C, N, O, and H absorb radiation at fundamental vibrational stretching frequencies (but most of these are not within the atmospheric windows). However, the overtones and combination bands of these absorption bands do occur within the near infrared (100-2500 nm) and in the ultraviolet (producing wings of the absorption in the visible) ranges. The absorptions occur mainly as functional groups, e.g., C-H 2nd overtones around 1200-1250 nm, which are commonly found in all organic molecules. The absorption features thus share these functional groups with closely spaced and overlapped absorption peaks, resulting in broadened composite absorption features. Thus, spectral measurements at any one wavelength will include some contribution from many biochemical compounds in leaves in approximate proportion to the concentration of that biochemical within the leaf. To sort out all this, one must use multiple measurements, even spectrally continuous spectrophotometric measurements, to determine the biochemical concentration from the composite spectral reflectance. While this is possible in the laboratory, where one can establish large sample sizes and a good calibration equation, it is only within the past 8 years that similar capability has existed from remote sensing platforms. The Jet Propulsion Lab developed the first spectrographic sensors with the Airborne Imaging Spectrometer, which produced spectrally continuous images with a spectral resolution of 10 nm. The spectral range was from 1200-2400 nm, i.e., 128 bands. They have since replaced this with the Airborne Visible Infrared Imaging Spectrometer (AVIRIS), which operates from Ames' ER-2 high altitude aircraft, acquiring data across the range of 400-2400 nm in about 228 spectral bands (Peterson *et al.*, 1988; Curran, 1989; Wessman *et al.*, 1988). These data have been used to estimate the concentration of lignin and nitrogen in forest canopies (Wessman *et al.*, 1988; Peterson and Running, 1989) and are being tested in sites throughout the United States and in Europe. The lignin concentration of the northern hardwood forests of Wisconsin's Blackhawk Island and Arboretum have been estimated using AIS data and lignin concentration has been mapped across the entire island

(about 1 Km²). A strong inverse relationship between lignin content of leaves and the annual rate of nitrogen mineralization was then used to map N-min over the island. N-min is key soil property related to ecosystem productivity, nitrogen turnover, and nitrogen losses as trace gases or in solution in these forests (Aber *et al.*, 1990).

Table 1 lists some of the "high" spectral resolution sensors planned for satellite operation during the Earth Observing System era. Much more research is needed to determine the precise measurement requirements and techniques and to develop a body of radiative transfer theory to support the systematic estimation of the biochemical content of plant canopies.

Simulation of Ecosystem Processes

As we continue to advance the state of the art in remote sensing, we can begin to use the variables estimated in models to predict ecosystem process rates. Since none of the process rates we would want to measure are directly sensible by remote sensing, we must use simulation models to predict them. A number of such models have recently been developed to do this. For example, we have developed a suite of integrated models that describe the ecosystem processes of carbon, nitrogen, and water cycling and routing, and the interactions between these cycles, to predict processes such as photosynthesis, respiration, nitrogen mineralization, nitrogen turnover, decomposition, evapotranspiration, and water yield. These models are mechanistic even though the remotely sensed variables are spatially variable (Peterson and Running, 1989). In this way, prediction of ecosystem processes that have in the past been restricted to smaller areas or extended statistically or in a highly aggregated way can now be predicted in a continuous fashion over very large regional landscapes (Running *et al.*, 1989).

The spatial variance must be considered carefully, however, since variations can be due to reflectance properties having little to do with the processes being predicted (i.e., variation in canopy coverage allowing background reflected radiation to introduce variance into remote sensing relationships such as LAI). One of the ways we have dealt with this is to look for scaling principles that are scale independent. This is opposed to taking a statistical sample and producing an estimation model of a process over a large region. We have used digital terrain data from the USGS to extract the stream network and associated drainage divides of all hillslopes in a mountainous landscape in an automated fashion. This system allows us to specify any stream order and generate irregular partitions of the landscape into various sized polygons. These polygons describe some of the larger scale environmental patterns which organize the landscape into more homogeneous units (Band *et al.*, 1991). Our initial tests with this system indicate that our predictions of ecosystem processes, such as annual amounts of evapotranspiration or net photosynthesis, are scale independent

on both structural (Lathrop and Peterson, 1991) and functional levels. Our research is continuing to investigate such scaling concepts and the potential for scaling laws of similarity that permit us to reliably relate when a small watershed (typically studied by ecologists) can tell us something about a much larger watershed and how to apply these principles.

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Molecular Phylogenies and Microbial Evolution

Phylogenies are often based on morphological analysis, but morphologies do not permit a universal phylogeny of life and are in general not useful for describing the evolutionary relatedness of microorganisms. Microbial diversity is perhaps best defined in terms of evolutionary relatedness, one measure of which results from the comparison of homologous gene or protein sequences. In addition to being homologous, an ideal molecule for a molecular phylogeny: 1) lacks lateral transfer; 2) provides an adequate number of nucleotides or amino acids to be statistically significant; and 3) is readily obtained and sequenced. Once sequences are determined, they are aligned so that each position along the molecule is compared to its homolog in other molecules. Relationship between any two organisms is calculated from the percent similarity between sequences. A matrix of similarities is then used to construct a phylogenetic tree that best fits the similarities between sequences.

Several molecules that have been used for universal molecular phylogenies are the ribosomal RNAs, elongation factors Tu and G, and the Fo and F' subunits of ATPase. The general picture that emerges from these analyses is that there are three primary lines of evolutionary descent, the archae, the bacteria, and the eucarya. It is also clear that the last universal ancestor of life was a fairly sophisticated organism, with a translational apparatus in place including ribosomal RNAs and proteins, elongation factors, and a prototype ATPase. In fact, one can begin to reconstruct the last universal ancestor by the comparative analysis reasoning that if a gene or gene product exists in each of the major lines of evolutionary descent, it must have been present in the last universal ancestor (barring lateral transfer). Iwabe *et al.* (1989) made significant advances in rooting a universal tree of life. By comparing genes that appear to have duplicated in the last universal ancestor, they arrived at a rooted phylogenetic tree showing that the archae and eucarya share a common ancestor after the divergence of the bacteria.

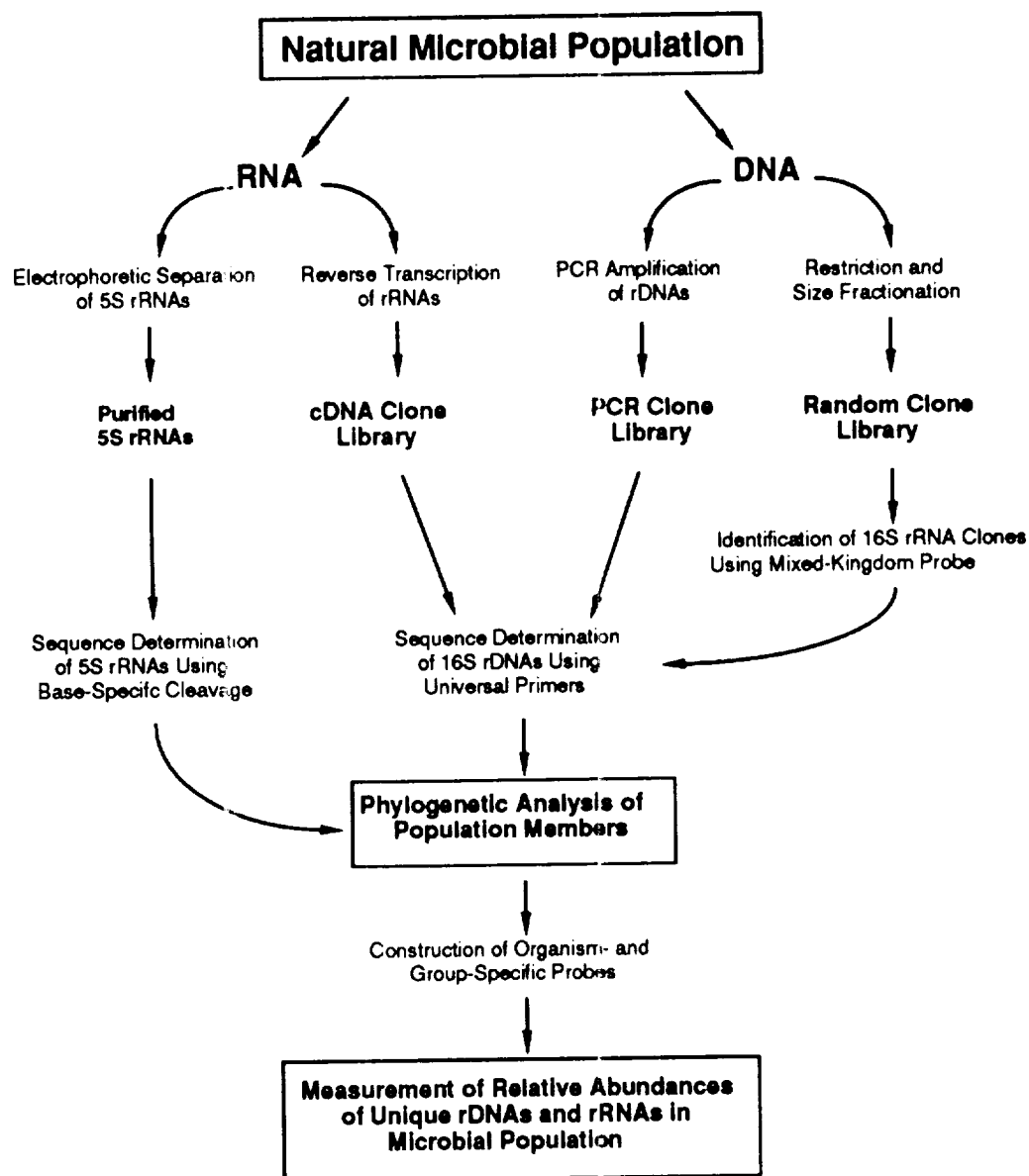
Identification and Quantification of Microorganisms in Natural Habitats Using rRNA-Based Probes

The identification of microorganisms in natural samples generally requires pure cultivation before conducting standard biochemical tests. This requirement has hampered studies of natural populations because of our inability to culture the majority of microorganisms present in any given environment. Additionally, varying efficiencies of cultivation of different organisms introduce uncertainties in the enumeration of microorganisms in environmental samples. To address these difficulties, molecular methods are being developed to identify and quantitate microorganisms in natural samples without the need for cultivation (Fig. 1). The methods utilize ribosomal RNA (rRNA) sequences for the identification of organisms and for the construction of nucleic acid probes for quantitative analysis.

The diversity of organisms in an environment can be determined by extracting total DNA from a sample, cloning size-fractionated DNA into phage lambda, and selecting recombinants containing rRNA genes. The rRNA inserts are then sequenced, and the sequence compared to a data base of known rRNA sequences to produce a tree of phylogenetic relationships.

Probes of several hundred nucleotides in length can then be transcribed from cloned and PCR-amplified rRNA genes, and hybridized to bulk DNA extracted from the environment. The hybridized probes are digested with nuclease S1, so that any probes lacking complementarity with the target are digested. Following S1 digestion, the full length probe is quantitated after electrophoretic separation. The remaining, intact probe is a representation of a particular rRNA gene (and so a particular organism) in the sample. In a second approach, fluorescently or isotopically labeled oligodeoxynucleotide probes are used for direct visualization of single cells. Ideally, the combination of these techniques permits the identification of microorganisms, the visualization of single cells, and a quantitative assessment of the abundance of specific microorganisms, all without the need for laboratory cultivation of the organisms.

Fig 1. Strategies for Obtaining rRNA Sequences from Natural Populations



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Transport Across the Cell Surface: Good Substrates and Bad

All materials (gases, growth substrates, antibiotics, and toxins) that find their way to the cellular cytoplasm must cross cell surface barriers, which in *Pseudomonads* consist of the outer membrane, an intermembrane periplasmic space, and the inner membrane. For antibiotics, *Pseudomonads* are well known for having effective outer membrane permeability barriers, leading to a high general level of antibiotic resistance. Antibiotics frequently cross the outer membrane through specific porin proteins, which also provide pathways for nutrient molecules smaller than 1,000 Daltons. The porins have limited substrate specificity but the outer membrane nonspecifically excludes larger molecules. Some antibiotics such as β -lactams function in the periplasmic space by associating with specific penicillin-binding proteins (PBP). Other antibiotics must cross the inner membrane, generally by poorly understood pathways thought to be designed for nutrient uptake. However, it is a general conclusion that most antibiotics are taken up by energy-dependent transport via specific transport proteins in the inner membrane. The inner membrane barrier is a "tight" membrane consisting of a phospholipid bilayer with numerous embedded transport proteins. The transport systems for different substrates (sugars, amino acids, vitamins, inorganic cations, and anions) are highly specific. Although *Pseudomonads* do not differ in principle from other Gram negative bacteria, we will survey what is known about the range of inner membrane transport systems, the functions of outer membrane porins, the uptake and barrier to uptake of clinically-important antibiotics, and the existence of membrane efflux systems that excrete (generally in an energy-dependent manner) metabolic waste products, antibiotics, and inorganic ions.

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Biochemistry and Molecular Biology of Bacterial Resistances to Toxic Heavy Metals

Bacterial plasmids contain specific genetically-determined resistances to a wide range of toxic heavy metals including Hg^{2+} , Cd^{2+} , Zn^{2+} , AsO_2^- , AsO_4^{3-} , CrO_4^{2-} , Cu^{2+} , Co^{2+} , Pb^{2+} , and other metals. Recombinant DNA analysis has been applied to cadmium, zinc, cobalt, nickel, arsenic, mercury, chromate, tellurium, and copper resistance systems. The first sequenced Cd^{2+} (and Zn^{2+}) resistance determinant governs a membrane efflux ATPase that assures a low level of cellular Cd^{2+} . The ATPase is one of the E1E2 class enzymes that include other bacterial membrane ATPases and those from animals and plants. It consists of two polypeptides and its synthesis is regulated by toxic cations. The second sequenced cadmium resistance system also confers resistances to zinc and cobalt, via a complex efflux pump determined by four polypeptides. The two cadmium resistance systems are not related at the sequence level. Still another ATPase governs arsenic resistance by maintaining low intracellular As(III) and As(V) levels. This complex is not related to the cadmium ATPase and consists of three polypeptides in *E. coli*, while only two have been identified in *Staphylococcus*. For chromate also, the first two sequenced systems are responsible for reduced cellular uptake but other bacterial systems exist that convert more toxic Cr(VI) to less toxic Cr(III). Eight mercury resistance systems have been sequenced. All contain genes for mercuric reductase, the enzyme that converts toxic Hg^{2+} to volatile and less toxic metallic Hg^0 . Four of these mercuric resistance systems also determine the enzyme organomercurial lyase, which cuts the Hg-C bond in methylmercury and phenylmercury, again converting more toxic into less toxic compounds. For each toxic heavy metal (with few exceptions) the resistances are specific. Understanding of the genetic and biochemical bases for these resistances is the first step toward the potential of using genetically engineered microbes for pollution control.

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Metallothioneins: Small Cadmium-Binding Proteins from Horses, Plants, and Bacteria

Metallothioneins are small (60 to 75 amino acids), high cysteine (up to 20-30% Cys residues) polypeptides, first isolated from animal sources 30 years ago and thought to be responsible for zinc storage and/or cadmium and copper resistances. Similar but evolutionarily independent metallothioneins were found in some yeast, and more recently in several higher plants and apparently in bacteria. The successful fishing for a metallothionein gene from a cyanobacterium (Robinson *et al.*, 1990) will be described in detail. Phytochelatin is not a protein; it is effectively poly-glutathione (γ -glutamyl-cysteinyl) $_n$ -glycine, with $n = 2$ to 11 . Phytochelatin has been studied extensively from plant materials during the last 5 years, and more recently from some yeast as well. There are no bacterial reports of phytochelatin, but the techniques used to seek these would not distinguish them from metallothionein.

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Recent Developments in Phylogeny of Eukaryotes

The widespread application of contemporary techniques for characterizing and manipulating DNA has revolutionized our understanding of macromolecular structures and evolutionary biology. Molecular approaches can now be applied to studies of ecology and population biology. Techniques are available for estimating biological diversity, studying population structure and measuring the flow of individual genes in interbreeding populations. The Molecular Evolution Center at the Marine Biological Laboratory offered two lectures to the Marine Ecology course and provided students the opportunity to use state of the art computer facilities and software for analysis of ribosomal RNA sequences. Dr. Mitchell Sogin offered a three hour lecture describing recent revelations about eukaryote evolution afforded by studies of ribosomal RNAs, as well as a demonstration of computer facilities for the analysis of molecular data. Multiple sequence alignment editors, phylogenetic analysis software, and access to the Ribosomal RNA Data Base in Urbana were demonstrated. The students were offered the opportunity to analyze unknown rRNA sequences isolated from naturally occurring populations. These sequences were compared to the aligned rRNA data base in order to identify their closest living relatives.

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An Overview of Nitrogen Cycling in Marine and Aquatic Systems

The first objective was to identify the most relevant nitrogen transformation processes and mechanisms. Among these were nitrogen fixation, denitrification, nitrification, and a large suite of other transformations. The second objective was to put the specific mechanisms and processes into the larger context of how they fit together into marine ecosystems. This was done using some case studies of coastal and deeper water marine environments. The third objective was to point out some of the most important applied implications of the above, and how knowledge of the nitrogen cycle is necessary for the management of coastal environments and water quality.

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Appendix: Abbreviations

β -ME	beta mercaptoethanol	
BB	bromphenal blue	
Ci	curies	
CIA	chloroform isoamyl alcohol	
conc.	concentration	
DEPC	diethylpyrocarbonate	
dil.	dilution	
DMF	dimethyl formamide	
DOM	dissolved organic material	
EPR	electron paramagnetic resonance	
GC	gas chromatography	
HB	hybridization solution	
IRMS	isotope ratio mass spectrometer	
ITC	isothiocyanate	
kb	kilobase	
kD	kiloDalton	
LAI	leaf area index	
Mb	megabase	
μ g	microgram	10^{-6} grams
μ l	microliter	10^{-6} liters
M	molar	
mM	milimolar	10^{-6} molar
ml	mililiter	10^{-3} liters
mg	miligram	10^{-3} grams
N	normal	
ng	nanogram	
nm	nanometer	
nM	nanomolar	
nmole	nanomole	
PBS	phosphate buffered saline	
pp't	precipitate	
RT	reverse transcriptase	
rpm	revolutions/minute	
soln.	solution	
T_d	dissociation temperature	
U	units	
UV	ultra-violet light	
V	volt	
W	watt	



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